Vol. 36, No. 2

June 1959

The Journal of Experimental Biology

EDITED BY

V. B. WIGGLESWORTH and J. A. RAMSAY

Contents

PAGE

Malcolm S. Gordon. Ionic regulation in the brown trout (*Salmo trutta* L.) 227

Malcolm S. Gordon. Osmotic and ionic regulation in Scottish brown trout and sea trout (*Salmo trutta* L.) 253

D. M. Steven. Studies on the shoaling behaviour of fish. I. Responses of two species to changes of illumination and to olfactory stimuli 261

Anne McLaren and Donald Michie. Superpregnancy in the mouse. 1. Implantation and foetal mortality after induced superovulation in females of various ages 281

Anne McLaren and Donald Michie. Superpregnancy in the mouse. 2. Weight gain during pregnancy. (With Plate 4) 301

R. McN. Alexander. The physical properties of the swimbladder in intact Cypriniformes 315

R. McN. Alexander. The densities of Cyprinidae 333

R. McN. Alexander. The physical properties of the isolated swimbladder in Cyprinidae . 341

R. McN. Alexander. The physical properties of the swimbladders of fish other than Cypriniformes 347

A. B. Gilbert. The composition of the blood of the shore crab, *Carcinus moenas* Pennant, in relation to sex and body size. II. Blood chloride and sulphate 356

T. E. Hughes, M.A. The cuticle of *Acarus siro* L. (= *Tyroglyphus farinae*) 363

Edward R. Baylor. The responses of snails to polarized light 369

A. Ibbotson and J. S. Kennedy. Interaction between walking and probing in *Aphis fabae* Scop 377

J. W. L. Beament. The waterproofing mechanism of arthropods. I. The effect of temperature on cuticle permeability in terrestrial insects and ticks 391

D. A. Parry and R. H. J. Brown. The hydraulic mechanism of the spider leg . . . 423

UNIVERSITY OF ILLINOIS
LIBRARY
JUL 17 1959
CHICAGO

Published for The Company of Biologists Limited

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 30s. net (U.S.A. $5.00). Subscription per volume 84s. (U.S.A. $14.00)

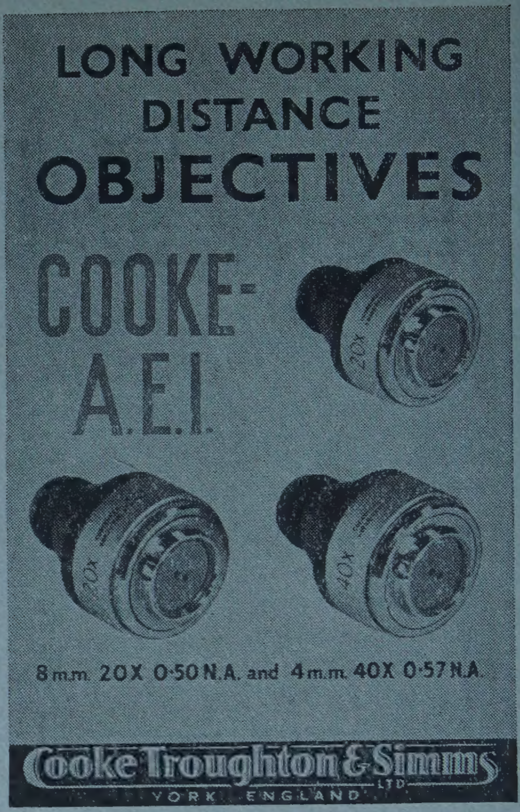
The Journal of
Experimental
Biology

INDEX
to
VOLS. 1–15

15s. net

*To members of the Society
of Experimental Biology, 7s. 6d.*

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD
LONDON, N.W.1



LONG WORKING
DISTANCE
OBJECTIVES

COOKE-
A.E.I.

8 m.m. 20X 0·50 N.A. and 4 m.m. 40X 0·57 N.A.

Cooke Troughton & Simms LTD.
YORK ENGLAND

THE JOURNAL OF PHYSIOLOGY

11 JUNE 1959. VOL. 146, NO. 3

VAUGHAN WILLIAMS, E. M. The effect of changes in extracellular potassium concentration on the intracellular potentials of isolated rabbit atria.

DEMPSHER, J., TOKUMARU, T. and ZABARA, J. A possible role of an inhibitory system in virus-infected sympathetic ganglia of the rat.

EVANS, M. H. and MCPHERSON, A. The effects of distension of the bladder on somatic reflexes in the cat.

CALNE, D. B. Pathways converging upon Purkinje cells in the frog's cerebellum.

BURNS, J. K. and HALE, A. J. The distribution of ribonucleic acid and some other substances in the adrenal cortex after administration of adrenocorticotrophin.

BORN, G. V. R. and GILLSON, R. E. Studies on the uptake of 5-hydroxytryptamine by blood platelets.

BURTT, E. T. and CATTON, W. T. Transmission of visual responses in the nervous system of the locust.

DAWES, G. S., MOTT, JOAN C. and SHELLEY, HEATHER J. The importance of cardiac glycogen for the maintenance of life in foetal lambs and new-born animals during anoxia.

HOOPER, K. C. and JESSUP, D. C. The distribution of enzymes destroying oxytocin and vasopressin in human placentae.

JOHN, E. R., TSCHIRGI, R. D. and WENZEL, BERNICE M. Effects of injections of cations into the cerebral ventricles on conditioned responses in the cat.

JENNER, F. A. and SMYTH, D. H. The excretion of phlorrhizin.

MCGRATH, MARGARET W. and THOMSON, M. L. The effect of age, body size and lung volume change on alveolar-capillary permeability and diffusing capacity in humans.

HALPERIN, M. H., MCFARLAND, R. A., NIVEN, J. I. and ROUGHTON, F. J. W. The time course of the effects of carbon monoxide on visual thresholds.

GELFAN, S. and TARLOV, I. M. Interneurones and rigidity of spinal origin.

Subscription price 80s. net per volume of 3 parts

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1

THE QUARTERLY JOURNAL OF MICROSCOPICAL SCIENCE

Joint Editors:

C. F. A. PANTIN, Sc.D., F.R.S. JOHN R. BAKER, D.Sc., F.R.S.

Third Series, No. 48

Contents of Volume 100, Part 1, March 1959

RYDER, M. L. Some Histochemical Observations on Amino-acids and Nucleic Acids in the Wool Follicle.

DEUTSCH, K. and SWANN, M. M. An Electron Microscope Study of a Small Free-living Amoeba (*Hartmanella astronyxis*).

JONES, J. C. A Phase Contrast Study of the Blood-cells in *Prodenia* Larvae (Order Lepidoptera).

KING, R. J. A Quantitative Study of the Cytoplasm of Ascites Tumour Cells in Mice.

KENNAUGH, J. An Examination of the Cuticles of two Scorpions, *Pandinus imperator* and *Scorpiops hardwickii.*

SRIVASTAVA, U. S. The Prothoracic Glands of some Coleopteran Larvae.

HINTON, H. E. The Function of Chromatocytes in the Simuliidae, with Notes on their Behaviour at the Pupal-adult Moult.

NICHOLS, D. The Histology of the Tube-feet and Clavulae of *Echinocardium cordatum.*

ANDERSON, D. T. The Embryology of the Polychaete *Scoloplos armiger.*

Subscription Price per Volume of 4 Parts £4. 4s. Single Parts 30s. net

OXFORD UNIVERSITY PRESS LONDON E.C.4

Journal of the Marine Biological Association of the United Kingdom

VOL. XXXVIII, NO. 2. JUNE 1959. 46*s.* net

J. E. MORTON. The habits and feeding organs of *Dentalium entalis.*

T. E. THOMPSON. Feeding in nudibranch larvae.

Z. KABATA. Ecology of the genus *Acanthochondria* Oakley, 1927 (Copepoda parasitica).

S. KRISHNASWAMY. Respiration in *Chondracanthus zei* de la Roche (Copepoda).

A. D. BONEY and E. D. S. CORNER. Application of toxic agents in the study of the ecological resistance of intertidal red algae.

C. M. YONGE, D.Sc., F.R.S. On the structure, biology and systematic position of *Pharus legumen* (L.).

L. R. FISHER and E. H. GOLDIE. The food of *Meganyctiphanes norvegica* (M. Sars), with an assessment of the contributions of its components to the vitamin A reserves of the animal.

A. H. STRIDE. A linear pattern on the sea floor and its interpretation.

IRENE MANTON. Electron microscopical observations on a very small flagellate: the problem of *Chromulina pusilla* Butcher.

D. ATKINS, D.Sc. The early growth stages and adult structure of the lophophore of *Macandrevia cranium* (Müller) (Brachiopoda, Dallinidae).

D. B. CARLISLE. Moulting hormones in *Palaemon* (=*Leander*) (Crustacea Decapoda). II. Differences between populations.

G. R. FORSTER. The ecology of *Echinus esculentus* L. Quantitative distribution and rate of feeding.

PATRICIA M. RALPH. Notes on the species of the Pteromedusan genus *Tetraplatia* Busch, 1851.

D. B. CARLISLE. On the sexual biology of *Pandalus borealis* (Crustacea Decapoda). I. Histology of incretory elements.

D. I. D. HOWIE. The spawning of *Arenicola marina* (L.). I. The breeding season.

A. D. WOODHEAD. Variations in the activity of the thyroid gland of the cod, *Gadus callarias* L., in relation to its migrations in the Barents Sea. I. Seasonal changes.

A. D. WOODHEAD. Variations in the activity of the thyroid gland of the cod, *Gadus callarias* L., in relation to its migrations in the Barents Sea. II. The 'dummy run' of the immature fish.

H. BARNES. Sea surface temperatures at Millport.

Abstracts of Memoirs.

Book Reviews.

Orders may be sent to your own bookseller
or to the publishers

Cambridge University Press
Bentley House
200 *Euston Road*
London, N.W. 1

The first work in the new Princeton University Press series, Investigations in the Biological Sciences

THE CELLULAR SLIME MOLDS

By JOHN TYLER BONNER

This book, by a recognized authority in the field, gives a concise summary of all the work that has so far been done on the cellular slime molds—those intriguing and enigmatic organisms which have a very wide geographical distribution but which are as yet relatively unknown. The principal emphasis is on the mechanics of development, and an attempt is made to fit knowledge of the slime molds into modern embryological views. The author discusses the relation of cellular slime molds to other organisms, the structure and life histories of different types of cellular slime molds, and experimental work not relating directly to problems of development. *Investigations in the Biological Sciences, No.* 1.

150 *pages. Illustrated.* $4

Order from your bookstore, or

***Princeton* UNIVERSITY PRESS**
PRINCETON, NEW JERSEY

FULL PARTICULARS OF THE

JOURNALS

PUBLISHED BY THE

CAMBRIDGE UNIVERSITY PRESS

MAY BE HAD FROM

THE MANAGER, CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD
LONDON, N.W. 1

VOLUME 36, No. 2 JUNE 1959

IONIC REGULATION IN THE BROWN TROUT (*SALMO TRUTTA* L.)

BY MALCOLM S. GORDON*

Department of Zoology, Yale University, and Woods Hole Oceanographic Institution†

(*Received* 11 *July* 1958)

INTRODUCTION

Studies of osmotic and ionic regulation in aquatic organisms may be divided into three major categories:

1. Descriptions of the overall patterns of osmotic and ionic regulation in the internal fluids of the organism when the organism is subjected to various physiologically or experimentally interesting osmotic and ionic stresses. Variations due to sex, time of year, temperature, age and size of organism, etc. should be considered in such descriptions.

2. Studies of the various pathways for movement of water, inorganic ions and other osmotically active substances into and out of the internal fluids of the organism. Such studies may be kinetic, biochemical, pharmacological, etc., and should include any internal movements of ions between the tissues and the internal fluids.

3. Studies of the mechanisms resulting in internal integration of the action of the various pathways (hormones and the central nervous system).

Teleost fishes have been studied to some extent in each of these ways, but a great deal more remains to be learned. Recent reviews of various aspects of the work that has been done are those of Beadle (1957), Black (1957), Fontaine (1954, 1956), Hoar (1953), Hoar, Black & Black (1951), Pickford & Atz (1957), D. C. W. Smith (1956) and von Buddenbrock (1956).

The euryhaline teleosts are of particular interest on account of their ability to survive in environments varying widely in salinity. The adult stages of several such forms have been investigated in some detail, though with greatly varying degrees of completeness.

Fairly complete Category 1 studies investigating the total osmotic pressure and chloride concentration of serum have been carried out on eels (*Anguilla spp.*: Duval, 1925; Boucher-Firly, 1935; Callamand, 1943; Koch, 1949; Fontaine & Koch, 1950, for summary), on the Atlantic salmon (*Salmo salar*: Benditt, Morrison & Irving, 1941; Fontaine, Callamand & Vibert, 1950), on the three-spined stickleback (*Gasterosteus aculeatus*: Gueylard, 1924; Heuts, 1942, 1945; Koch and Heuts, 1942, 1943), and on the ten-spined stickleback (*Pungitius pungitius*: Heuts, 1943).

Much less complete studies of osmotic pressure and/or chloride have been carried out on a number of other forms. Most of this work consists simply of measurements of blood concentration in groups of fish acclimatized to fresh water and to salt water, no attention having been given to temperature, season, etc. The forms investigated include brown trout (*Salmo trutta*: Dekhuyzen, 1905; Parry, 1958), rainbow trout (*Salmo gairdneri*: Busnel, 1942; Parry, 1958), chinook salmon (*Oncorhynchus tchawytscha*: Greene, 1904, 1926), various Japanese salmonids (*Oncorhynchus spp.* and *Salvelinus spp.*: Kubo, 1953), Arctic char (*Salvelinus alpinus*: Gordon, 1957), shad (? *Alosa sp.*: Fontaine, 1930) and killifish (*Fundulus heteroclitus*: Burden, 1956; Garrard, 1935).

---

* Present address: Department of Zoology, University of California, Los Angeles 24, California.

† Contribution number 989 from the Woods Hole Oceanographic Institution.

Eels and Atlantic salmon are the only forms for which any significant amount of information is available on sodium, potassium and phosphorus concentrations in serum—and the data are very sparse even for these. Sodium and potassium were studied in the eel by Drilhon (1943), phosphorus by Fontaine & Firly (1932), Boucher-Firly (1935) and Ichikawa (1955). Sodium in the Atlantic salmon was studied by Fontaine, Callamand & Vibert (1950).

Some additional information available for the brown trout includes: studies of survival in hypertonic environments (D.C.W. Smith, 1956; Parry, 1958); values for the concentrations of most of the major inorganic ions in the serum of trout in fresh water in summer (Phillips & Brockway, 1958); and values for sodium and potassium in the serum of trout in fresh water in winter (Spalding, in Jones, 1956).

Many fewer Category 2 studies have been carried out on fishes, much less on euryhaline forms. Most of the work which has been done is thoroughly discussed in the reviews listed above and elsewhere. It should be noted, however, that with the exception of the somewhat fragmentary work of Mullins (1950) on *Gasterosteus* no work has been done on estimations of ion flux rates along the various pathways, or on the detailed mechanism of salinity adaptations, using modern methods.

A further omission, from the older literature as well as the new, has been a consideration of the possible significance for regulation of blood concentrations of internal ion movements between various tissues, especially the muscles, and the blood. The only references to this topic known to me are in the very partial studies of Drilhon (1937) and Callamand, Fontaine, Olivereau & Raffy (1951) on the eel. Drilhon suggests, and the data of Callamand *et al.* seem to lend some support to her suggestion that the muscles of the eel act as a reservoir of salts when the fish is in salt water.

The reader is referred to the reviews listed above for summaries of the Category 3 work carried out on euryhaline fishes.

The present paper falls into both Categories 1 and 2. The form studied was the brown trout (*Salmo trutta* L.), a euryhaline salmonid fish which is often anadromous. Information on the differences between sea-run and strictly fresh-water brown trout is given by Gordon (1959).

The main purpose of the work was to establish in some detail the overall pattern of normal variation (and the changes occurring in this pattern following osmotic stresses similar to those which would be encountered during migration to the sea) of the quantitatively most important inorganic constituents of the blood and muscles of brown trout. With this end in view, sexually mature brown trout within a limited size range were acclimatized for varying periods of time to fresh water and to half- and to full-strength sea water. Such acclimatization experiments were repeated three times during the course of a year at one temperature, and once at a different temperature. Blood and muscle samples were taken from groups of fish at various times during the acclimatization process. These were analysed for the following components:

Serum: freezing-point depression, ° C. (Δ); chloride, m-equiv./l. (Cl_s); sodium, m-equiv./l. (Na_s); potassium, m-equiv./l. (K_s); and total phosphorus, mg./l. (P).

Muscles: total solids, g./kg. wet weight; chloride, m-equiv./kg. wet weight (Cl_m); sodium, m-equiv./kg. wet weight (Na_m); and potassium, m-equiv./kg. wet weight (K_m).

The results of this overall (Category 1) survey will be presented first, consideration being given to the effects of season and temperature. The implications of these results will then be considered under the following headings: water and ion movements between the muscles and the blood (a test of Drilhon's (1937) ion store hypothesis—Category 2), and ecological implications.

MATERIAL AND METHODS

Animals

Sexually mature brown trout 18–24 cm. in standard length were obtained from two hatcheries in the states of Connecticut and Massachusetts respectively, the majority coming from the Massachusetts hatchery. All handling necessary was done as gently as possible, usually by means of smooth linen nets. The fish were transported from the hatcheries to the laboratory in 20-gal. galvanized iron garbage cans painted on the inside with black asphaltum varnish. Considerable mortality occurred in preliminary experiments using unpainted cans.* The fish were not fed for several days prior to, and during, the experiments. In order to prevent them from fighting the fish were maintained separately in smaller enclosures within the large tanks used.

Hatchery water temperatures varied seasonally from about 2° to 24° C. Experimental series involving temperatures significantly different from these hatchery temperatures were preceded by a gradual thermal acclimatization of the fish over 12–24 hr. The fish were maintained in aerated hatchery water during this period.

Experimental design

All experimental series followed the same pattern. Control fish were sampled (i) directly from aerated fresh water in the hatchery tanks, (ii) in the laboratory after having undergone the same thermal acclimatization as the experimentals (zero time controls), and (iii) at intervals during the experimental period. They were handled and otherwise treated as experimentals. Experimental fish were transferred from fresh water (salinity < 0·2‰) directly to aerated running half-strength sea water (salinity 15 ± 2‰) or to aerated running full-strength sea water (salinity 31·5 ± 0·5‰). Groups of fish were sampled at intervals up to ten days following transfer. In the 20° C. series, where direct transfer to full sea water proved fatal to all fish within 15–30 hr., fish acclimatized to half sea water for 10 days were further transferred to full sea water and samples were taken up to 10 days following the second transfer. The work of Boucher-Firly (1935) on *Anguilla*, that of Gordon (1959) and Parry (1958) on *Salmo trutta*, and the present results indicate that ten days is enough time for at least all major changes in internal concentrations to have taken place.

---

* Unpainted galvanized tubs were used by Gordon (1957) for handling Arctic char at Churchill, Manitoba. It seems likely that much of the mortality encountered in that work was due to this cause.

Experimental series were designed to demonstrate (i) seasonal effects, if any, at constant temperature, and (ii) temperature effects within one season. One preliminary experiment was done using fish from the Connecticut hatchery, but all other experiments were done with Massachusetts fish. Experiments with Massachusetts fish were as follows:

1. Seasonal effects at constant temperature (20 ± 2° C.):

(*a*) controls; half and full sea-water transfers in the breeding season (September–November). (*b*) controls; half and full sea-water transfers in winter and spring (February–April). (*c*) controls; half and full sea-water transfers in summer (June–August).

2. Temperature effects within one season (winter—a few data from the breeding season were also obtained):

(*a*) controls and full sea-water transfers at 10 ± 2° C. (*b*) controls; half and full sea-water transfers at 20 ± 2° C. (Exp. 1 (*b*)).

Details of the experimental protocols are given in Table 1.

The preliminary experiment using Connecticut fish included fresh-water controls and transfers to seven-eighths sea water (salinity 28‰) at 10° C. in February. Its principal purpose was to test technique and it was continued for only three days.

Complete series of serum and muscle analyses were done on all fish in Exps. 1 (*a*), (*b*), 2 (*a*), (*b*), in the fresh-water controls in 1 (*c*) and in the preliminary experiment. Determinations of muscle solids only were done on the half and full sea-water transfers in 1 (*c*).

Photoperiod was uncontrolled in all experiments, but the fish were obtained from outdoor tanks at the hatchery in which they had been exposed to the seasonal variations in light.

Analytical techniques

Blood samples. 1·0–2·0 ml. of whole blood were usually easily obtained by heart puncture with a 1 ml. tuberculin syringe and a fine (no. 22) needle. In cases where difficulties were encountered, samples were taken with the same syringe from the caudal artery and vein dorsal to the anterior edge of the insertion of the anal fin. Grafflin (1935) and Forster (1953) have shown that samples obtained in these two

Table 1. *Protocols of experiments with Massachusetts fish*

(August 1956 to August 1957.)

Starting date	Hatchery water temp. (° C.)	No. of fish	Samples obtained (state of acclimatization, no. of fish in sample)
1 (*a*) Breeding season series—20 ± 3° C.			
4. ix. 56	16	20	20 in ½ SW: 2 hr. 2; 4 hr. 2; 8 hr. 2; 12 hr. 2; 24 hr. 4; 48 hr. 2; 72 hr. 3. 3 dead.
20. ix. 56	11	22	14 in ½ SW: 8 hr. 2; 48 hr. 4; 240 hr. 7. 1 dead. 8 in FW: 72 hr. 4; 240 hr. 3. 1 dead.
2. x. 56	12	26	6 in FW: 0 hr. 6. 20 in SW: 2 hr. 2; 4 hr. 2; 8 hr. 2; 12 hr. 2; 24–30 hr. 9. 3 dead.
8. x. 56	12	12	9 in ½ SW. 7 survivors after 240 hr. to SW: 12 hr. 2; 48 hr. 5. 3 in SW: 3 hr. 1; 18 hr. 1. 1 dead.
10. x. 56	11	6	Hatchery controls—6.
24. x. 56	13	9	9 in ½ SW. 9 survivors after 240 hr. to SW: 130 hr. 2; 240 hr. 6. 1 dead.
1 (*b*) Winter and spring series—20 ± 2° C.			
18. ii. 57	3	15	15 in FW: 0 hr. 6; 72 hr. 6. 3 dead.
26. ii. 57	8	9	9 in SW: 8 hr. 4. 5 dead.
4. iii. 57	4	18	18 in SW: 16 hr. 3; 21–27 hr. 6. 9 dead.
8. iii. 57	6	18	18 in ½ SW: 8 hr. 4; 16 hr. 4; 24 hr. 6. 4 dead.
11. iii. 57	4	16	7 in FW: 7 dead. 9 in ½ SW: 72 hr. 6. 3 dead.
18. iii. 57	8	18	9 in FW: 240 hr. 1. 8 dead. 9 in ½ SW: 240 hr. 5. 4 dead.
28. iii. 57	9	18	9 in FW: 9 dead. 9 in ½ SW: 5 survivors after 240 hr. to SW: 240 hr. 1. 4 dead.
29. iii. 57	10	6	Hatchery controls—6.
1 (*c*) Summer series—20 ± 2° C.			
17. viii. 56	18	6	Hatchery controls—6.
27. v. 57	16	9	9 in ½ SW. 7 survivors after 240 hr. to SW: 240 hr. 3. 4 dead.
5. vi. 57	16	9	9 in ½ SW: 240 hr. 5. 4 others to SW: 4 dead.
21. vi. 57	20	9	9 in ½ SW. 9 survivors after 240 hr. to SW: 9 dead.
25. vi. 57	20	5	Hatchery controls—5.
23. vii. 57	21	10	5 in ½ SW: 24–29 hr. 5. 5 in SW: 5 dead.
5. viii. 57	24	5	5 in SW: 12–16 hr. 5.
2 (*a*) Winter series—10 ± 2° C.			
11. ii. 57	3	24	6 in FW: 0 hr. 6. 18 in SW: 18 hr. 4; 24 hr. 6; 52 hr. 4 (dying); 72 hr. 4.
13. ii. 57	2	9	9 in SW: 8 hr. 4; 72 hr. 2; 240 hr. 1. 2 dead.
18. ii. 57	3	8	8 in SW: 240 hr. 1. 7 dead.
26. iii. 57	9	12	12 in FW: 72 hr. 6; 240 hr. 6.

ways do not differ significantly with respect to the concentrations being considered here.

Initially, approximately 0·2 mg. of the sodium salt of heparin was added to each sample to prevent clotting. Since this was generally ineffective, most of the samples (> 95 %) were simply allowed to clot. No significant differences were found with respect to the quantities studied between duplicate series of heparinized and unheparinized samples.

The samples were placed in centrifuge tubes, the clots and corpuscles were centrifuged down, and the clear serum was pipetted off. The serum samples were placed in acid-cleaned vials, stoppered tightly with paraffined corks, and frozen until analysed. Variable amounts of haemolysis occurred in different samples. This will be discussed further below (p. 233).

Muscle samples. Four samples of 150–350 mg. wet weight (usually 200–250 mg.) were excised from the dorsal muscle mass of each fish on the left side somewhat below the dorsal fin. Care was taken to remove all skin and adhering scales. Total muscle solids were determined by placing these samples on tared aluminium pans, weighing them wet, then re-weighing them after 24 hr. in an oven at 105° C. (samples from other parts of single fish gave identical results). It will be seen that there were only small seasonal variations in this total concentration, and for this reason possible fluctuations in fat content are considered insignificant. The dried muscle samples were stored in stoppered Pyrex test tubes (cleaned with acid and detergent) until analysed. Agreement between quadruplicate determinations of total solids was ±2 g./kg. about the mean.

Chemical methods (only Pyrex glassware used throughout). Serum Δ was determined on approximately 1 mm.^{3} of serum by the method of Kinne (1952), somewhat modified by Werntz (1957). Precision was ±0·02° C.

All other analyses were carried out on aliquots of serum or dried muscle samples digested at 100° C. for about 30 min. in 0·5–1·0 ml. concentrated nitric acid plus 0·5–1·0 ml. 30% hydrogen peroxide (both Analytical Reagent grade). A drop of caprylic alcohol was added to each muscle sample to prevent foaming; serum samples that foamed were treated similarly. Appropriate standards (both pure and additions to samples) and blanks were run. Duplicate analyses were made for each substance in each tissue of each fish. The deviation of the two observations from their mean value was recorded for many hundreds of such duplicates, and the average deviation is given below as the figure for precision of each analytical method used. Unless noted otherwise serum concentrations are expressed as per litre, muscle concentrations as per kilogram wet weight.

Chloride. Analyses were made by Volhard titration using the method of Van Slyke (1923) as modified by H. W. Smith (1930). Heilbrunn & Hamilton (1942) and Shenk (1954) have criticized this technique as applied to muscle analyses. The data presented by these authors indicate that, for muscle samples, the Van Slyke technique gives incorrect figures for total muscle chloride. However, figures given by Conway (1935, 1957) and others for inorganic muscle chloride, determined in a variety of well-tested ways (including some very similar to that used by Heilbrunn and Hamilton), agree quite well with those obtained by the Van Slyke technique. Primarily for this reason the modified Van Slyke procedure has been used throughout the present work. Serum samples were 0·100 ml., muscle samples were two of the dried pieces described above. Precision for serum analyses was +2–3 m-equiv./l., for muscle analyses ±1 m-equiv./kg.

The chloride space (assuming all muscle chloride is extracellular) has been used as an estimate of extracellular volume in the muscles throughout this work. This is known to be somewhat in error, but many studies (cf. Conway, 1957) have shown that this approximation generally results in an error in the volume calculated (compared to inulin space) of only 5–10% in the muscles of cold-blooded animals. The alternative method of calculating the extracellular volume by assuming a Donnan equilibrium across the fibre membranes (Boyle & Conway, 1941; Conway, 1957) gives significantly lower (by 40%) values than the chloride space in the present material. This would be a matter for concern if it were not for the relatively poor precision of this latter method, also for the evidence that has recently been accumulating that the basic assumption may be unjustified—at least in frog muscle (Shaw, Simon & Johnstone, 1956; Shaw, Simon, Johnstone & Holman, 1956; Simon, Shaw, Bennett & Muller, 1957; Stephenson, 1957). No estimate has been made of the blood space within the muscles.

Sodium. Analyses were made at 589 mμ with a Beckman DU quartz spectrophotometer with a flame photometer attachment using an oxygen-acetylene flame. Addition standards showed that no corrections of concentrations determined were needed to compensate for flame background changes due to the influence of the nitric acid or other materials present in the serum samples (compare Berry, Chappell & Barnes, 1946; Parks, Johnson & Lykken, 1948). A correction of −10% for muscle concentrations was indicated by similar standards, however. All figures presented here contain this correction. Excess phosphate (as ammonium hydrogen phosphate) was added to all samples for flame photometry to avoid possible variable effects of low phosphorus concentrations on sodium emission such as were found by Ramsay, Brown & Falloon (1953) (Margoshes & Vallee, 1956, however, found no such effects). Serum samples were 0·200 ml., digested, then diluted quantitatively with glass-distilled water 125 times; muscle samples were aliquots of quantitative dilutions of the nitric acid and peroxide digests of the two dried samples not used for the chloride analyses. Precision for serum analyses was ±1–2 m-equiv./l., for muscle analyses ±0·6 m-equiv./kg.

Potassium. Analyses were made at 771 mμ by flame photometer on the same samples as used for sodium. Precision for serum analyses was ±0·06 m-equiv./l., for muscle samples ±2 m-equiv./kg. Again, addition standards indicated no correction was needed for serum concentrations determined, but a correction of +5% was necessary for muscle concentrations. All figures presented here contain this correction.

Phosphorus. Analyses were made on aliquots of the quantitative dilutions of the serum samples used for flame photometry. The method of Chen, Toribara & Warner (1956), with ascorbic acid as the reducing agent, was used. Precision was ±5 mg./l.

Effects of haemolysis

The variable amount of haemolysis occurring in serum samples was mentioned above. It would be expected that rupture of some red corpuscles would influence serum potassium and phosphorus concentrations. One might expect that higher potassium and phosphorus concentrations in any one group of fish would be correlated with greater amounts of haemolysis. A general correlation of this sort did exist, but moderate degrees of haemolysis had no significant influence on these concentrations. All values for these concentrations in strongly haemolysed samples (these constituted less than 2% of all samples) have been excluded from the figures here presented.

RESULTS

Sexual and seasonal effects

Two general points need consideration before beginning a detailed survey of the results obtained for the specific concentrations studied. These are the questions of sexual differences and seasonal effects.

No consistent sexually correlated differences have been found for any of the concentrations studied in the present work. Comparisons between values for all concentrations studied in all males and all females in both series of experiments between September and April showed no differences statistically significant at the 5% level by '*t*' test. Breeding and non-breeding males and females considered separately also showed no differences. In all the following, therefore, the sexes will be considered together.

No seasonal differences in survival of experimental fish that could not be attributed to one or more of a number of extraneous influences were noted between September and April. A decrease in survival of fish gradually acclimatized to full sea water at 20° C. was noted during the summer, however. This may have been real (see p. 244).

P_s (Table 7) and Na_m (Table 5) were the only quantities measured showing consistent statistically significant seasonal variations between September and April. Excepting these two quantities, therefore, the results of both the breeding-season and the winter–spring series of experiments at 20° C. will be considered together. The seasonal variations in P_s and Na_m will be discussed in later sections.

This absence of seasonal variation was only apparent during the period September to April. The markedly different situation existing during the summer will be described below (p. 241).

Experiments at 20° C., September to April

Survival

Table 1 shows that great variations occurred in the mortality due to other than experimental causes in different groups of fish within any one experimental series, as well as between experimental series. As stated above, this was due to many extraneous factors. Allowing for the uncertainty due to these factors, it is probably still accurate to say that trout acclimatized to half-strength sea water, or to half-strength and then to full-strength sea water at 20° C. between September and April survived in the laboratory about as well as did fresh-water controls.

Serum concentrations

Fig. 1 shows the pattern of changes in Δ following isothermal transfers to fresh water, half sea water and full sea water (both directly from fresh water and after ten days' acclimatization to half sea water) at 20° C. between September and April. Cl_s and Na_s varied in almost identical fashions.

The numerical data from these experiments are summarized in Tables 2–4. Zero time controls are separated from other controls in these tables and all following tables. Zero time fish had apparently not completely recovered from the effects of handling, thermal acclimatization, etc. (cf. P, p. 238).

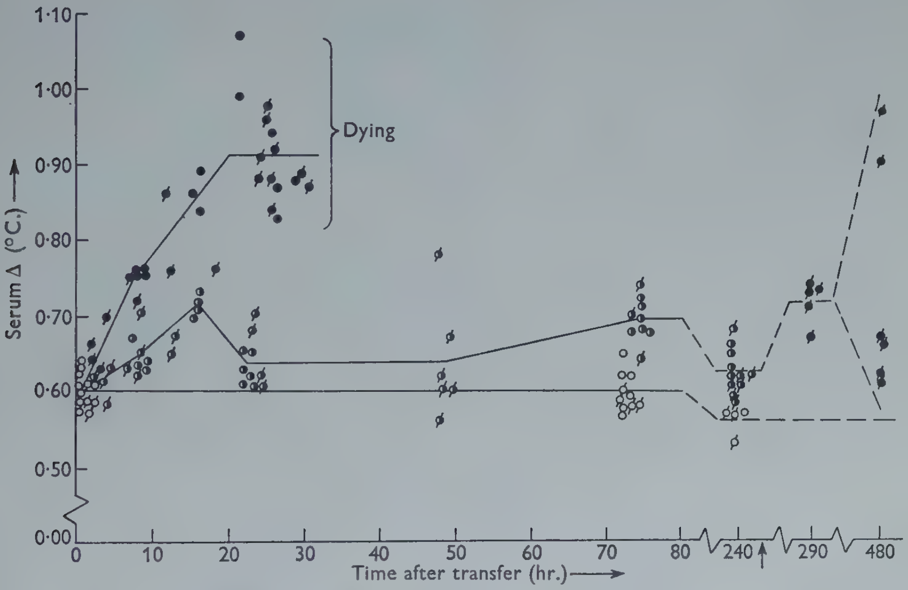


Fig. 1. Time course of changes in serum Δ in Massachusetts hatchery fish following transfers from fresh water to half-strength and to full-strength sea water at 20° C. between September and April. Open circles (○) are fresh-water controls, half-black circles (◑) half sea-water transfers, black circles (●) full sea-water transfers. Symbols with diagonal marks (e.g. ∅) represent fish sampled during the breeding season. The arrow at 240 hr. indicates transfer from half to full sea water. Ruled lines join the means for the various groups. Unless noted otherwise, the symbols used in the other figures have similar meanings.

In the control fish maintained in fresh water $\Delta = 0{\cdot}59°$ C. and no significant changes occurred (240 hr. controls are insignificantly different from 72 hr. controls at the 1 % level by 't' test). The shock of handling the fish, and also their being starved for the experimental period, therefore had no effect on their overall osmoregulatory abilities assessed in this way.

In the first few hours following transfer from fresh water to half-strength and to full-strength sea water Δ increased almost linearly with time, the rates of increase varying approximately in proportion to the initial concentration gradients established between the fishes' blood and their external environments.

In half sea water the concentration continued to increase for 10–15 hr., after which period it became more or less stabilized at a new, higher level, Δ = 0·69° C. after 72 hr.

Table 2. *Changes in serum Δ and total muscle solids following application of osmotic stresses*

(September–April.)

State of acclimatization	Serum Δ (° C.) $[\bar{x} \pm$ S.E. (N)]	Muscle solids (g./kg.) $[\bar{x} \pm$ S.E. (N)]
	20° C. experiments	
FW, 0 hr.	0·604±0·005 (12)	210·2±1·2 (12)
FW, 72–240 hr.	0·587±0·008 (14)	209·7±2·3 (14)
½ SW, 16 hr.	0·715±0·006 (4)	233·2±4·3 (4)
½ SW, 24 hr.	0·637±0·010 (10)	232·0±2·7 (25)
½ SW, 48 hr.	0·638±0·032 (6)	
½ SW, 72 hr.	0·693±0·010 (9)	
½ SW, 240 hr.	0·625±0·008 (11)	210·1±2·0 (12)
SW, 21–30 hr.	0·914±0·016 (15)	279·7±2·7 (15)
½ SW, 240 hr., SW, 47 hr.	0·716±0·013 (5)	239·2±7·5 (5)
½ SW, 240 hr., SW, 240 hr.	0·738±0·064 (6)	225·1±7·1 (7)
	10° C. experiments	
Hatchery controls	0·563±0·003 (12)	205·2±2·0 (12)
FW, 0 hr.	0·582±0·010 (6)	207·3±4·8 (6)
FW, 72–240 hr.	0·555±0·006 (11)	201·8±3·5 (12)
SW, 24–72 hr.	0·747±0·013 (12)	252·2±4·5 (12)
SW, 240 hr.	0·62, 0·81 (2)	217, 233 (2)

Table 3. *Changes in serum and muscle chloride following application of osmotic stresses*

(September–April.)

State of acclimatization	Cl_s (m-equiv./l.) $[\bar{x} \pm$ S.E. (N)]	Cl_m (m-equiv./kg.) $[\bar{x} \pm$ S.E. (N)]	$\mathrm{Cl}_m/\mathrm{Cl}_s \times 10^3$
	20° C. experiments		
FW, 0 hr.	129·2±1·3 (12)*	8·2±0·3 (12)	64 (59–68)†
FW, 72–240 hr.	140·5±1·1 (14)*	9·6±0·6 (14)	68 (60–77)
½ SW, 24 hr.	153·5±3·1 (10)	10·7±0·9 (10)	70 (58–82)
½ SW, 72 hr.	164·7±2·9 (9)	9·6±0·6 (9)	58 (51–66)
½ SW, 240 hr.	148·2±2·3 (12)	9·3±0·5 (12)	63 (56–70)
SW, 21–30 hr.	203·6±3·3 (15)	14·0±1·1 (15)	69 (58–80)
½ SW, 240 hr., SW, 47 hr.	170·4±1·6 (5)	13·4±1·0 (5)	79 (67–91)
½ SW, 240 hr., SW, 240 hr.	179·1±11·5 (7)	12·5±1·5 (6)	71 (54–88)
	10° C. experiments		
Hatchery controls	130·0±1·3 (12)	7·8±0·5 (12)	62 (52–72)
FW, 0 hr.	131·3±1·2 (6)	7·5±0·5 (6)	57 (50–65)
FW, 72–240 hr.	128·3±1·2 (11)	8·8±1·5 (6)	69 (45–92)
SW, 24 hr.	171·9±3·2 (12)	10·2±0·6 (6)	60 (52–66)
SW, 72 hr.		8·7±0·7 (6)	50 (42–59)
SW, 240 hr.	146, 189 (2)	9, 17 (2)	62, 90

* The difference in means between these two groups of controls is due to a lag in the response of the fish to temperature change. Temperature of the hatchery water at the times these experiments were carried out was near 10° C. (See Material and Methods section.)

† Approximate 95 % confidence interval. (See p. 241.)

Table 4. *Changes in serum sodium following application of osmotic stresses*

(September–April.)

State of acclimatization	Na_s (m-equiv./l.) [$\bar{x} \pm$ S.E. (N)]
20° C. experiments	
FW, 0 hr.	148·1 ± 1·7 (12)
FW, 72–240 hr.	149·3 ± 1·4 (13)
½ SW, 24 hr.	165·4 ± 1·9 (10)
½ SW, 72 hr.	179·0 ± 2·3 (9)
½ SW, 240 hr.	162·1 ± 2·8 (12)
SW, 21–30 hr.	231·1 ± 4·7 (15)
½ SW, 240 hr., SW, 47 hr.	182·6 ± 1·7 (5)
½ SW, 240 hr., SW, 240 hr.	194·0 ± 15·2 (6)
10° C. experiments	
Hatchery controls	146·9 ± 1·8 (12)
FW, 0 hr.	147·5 ± 1·7 (6)
FW, 72–240 hr.	145·4 ± 1·1 (12)
SW, 24–72 hr.	188·8 ± 4·5 (12)
SW, 240 hr.	154, 190 (2)

Table 5. *Changes in total sodium in the muscles following application of osmotic stresses*

(September–April.)

State of acclimatization	Na_m (m-equiv./kg.) [$\bar{x} \pm$ S.E. (N)]
20° C. experiments—breeding season	
FW, 0 hr.	14·4 ± 1·4 (6)
FW, 72–240 hr.	18·8 ± 1·2 (7)
½ SW, 24 hr.	16·0 ± 1·4 (4)
½ SW, 72 hr.	13·6 ± 1·6 (3)
½ SW, 240 hr.	17·9 ± 0·8 (7)
SW, 21–30 hr.	26·6 ± 1·7 (9)
½ SW, 240 hr., SW, 47 hr.	19·6 ± 1·6 (5)
½ SW, 240 hr., SW, 240 hr.	13·6 ± 1·7 (6)
20° C. experiments—spring	
FW, 0 hr.	8·2 ± 0·2 (6)
FW, 72–240 hr.	12·0 ± 1·3 (7)
½ SW, 24 hr.	10·7 ± 1·1 (6)
½ SW, 72 hr.	12·1 ± 0·6 (6)
½ SW, 240 hr.	9·7 ± 0·8 (5)
SW, 21–30 hr.	25·0 ± 1·9 (6)
10° C. experiments—spring unless noted	
Hatchery controls	7·9 ± 0·3 (6)
Hatchery controls, breeding season	11·2 ± 0·8 (6)
FW, 0 hr.	9·0 ± 0·5 (6)
FW, 72 hr.	8·6 ± 2·0 (6)
SW, 24 hr.	13·4 ± 1·0 (6)
SW, 72 hr.	11·6 ± 0·9 (6)
SW, 240 hr.	9·4, 17·1 (2)

In full sea water the concentration continued to increase for approximately 20 hr., after which period it may well have begun to level off at about $\Delta = 0{\cdot}91°$ C., some 56% above the fresh-water level. The fish did not survive long enough for this to be certain, however. Many were already dead after 24 hr. and all were at least moribund after about 30 hr.

The fish in half sea water survived with relatively little mortality for ten days. After this period they had all made some return toward their original fresh-water concentrations, though they still averaged 6% above controls (difference statistically significant at the 1% level). Such acclimatized fish transferred to full sea water again increased in concentration, but only to $\Delta = 0{\cdot}72°$ C., which is about the same value as that reached after transfer from fresh water to half sea water. As far as their ability to regulate blood concentration was concerned, full sea water had essentially become half sea water for these fish.

Ten days in full sea water were now survived with comparatively little difficulty, though the fish often became very emaciated and some died, apparently of starvation and tank injuries. After 10 days in full sea water the relative uniformity of response demonstrated to previous stresses was no longer present. Variability for serum concentration increased by a factor of 4–6. Serum concentrations equal to those found in dying fish 24 hr. after direct transfer from fresh water to full sea water were now encountered in seemingly healthy fish. Other healthy fish had returned all the way to their original fresh-water concentration levels. The significance of these observations for the present work is discussed below (p. 247).

The patterns shown by Cl_s and Na_s were, as noted above, the same as that for Δ. Assuming unit activity for the ions, chloride contributed a quite constant 40–45% of the Δ of the blood, sodium 45–49%. These relations are shown in Fig. 2, in which Cl_s and Na_s are plotted against Δ. The regression lines for these relations, calculated by the method of least squares, are included in Fig. 2. The equations for these lines are: $Cl_s = 211{\cdot}9\,\Delta + 13{\cdot}4$; $Na_s = 234{\cdot}6\,\Delta + 14{\cdot}7$ (using absolute values for Δ). The slopes of the lines are statistically insignificantly different from one another ($P = 0{\cdot}4$, by 't' test). Correlation coefficients for the two sets of data are: Cl_s, Δ: 0·654; Na_s, Δ: 0·704.

Changes in K_s and P following the various transfers were small and virtually all statistically insignificant (Tables 6 and 7). Such variations as did occur were only generally related to the other concentration changes already described. K_s on the average was regulated very well on a low level.

Within the separate experimental series P remained essentially constant. The only variation encountered which appears biologically significant is the seasonal one (see p. 245).

For brief periods following handling or some other sort of shock P often increased somewhat. This effect is illustrated in Table 7 by the higher and more variable phosphorus concentrations found in all groups of zero time controls as compared with controls which had been kept for some time under constant environmental conditions.

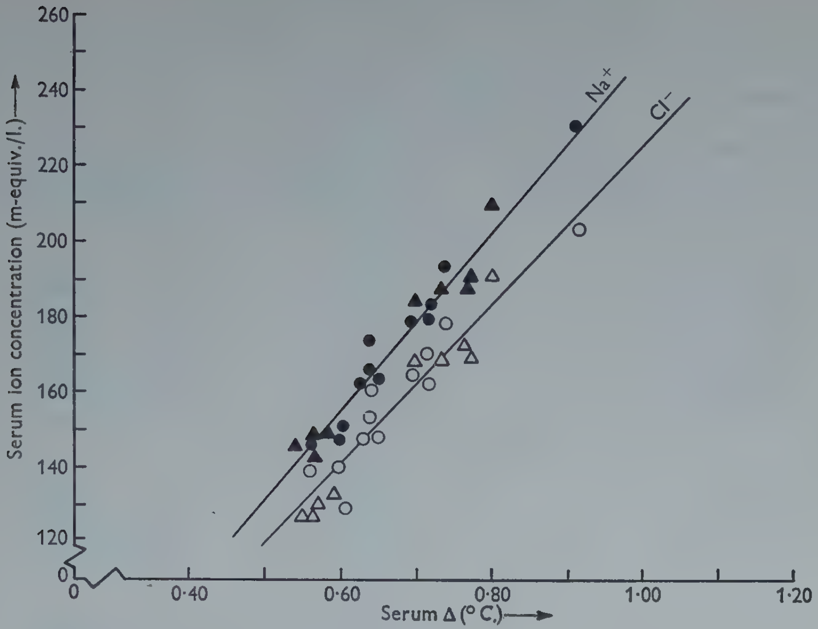


Fig. 2. Relations between Cl_s and Na_s and Δ. For clarity the only points plotted are those representing the mean values for these quantities in the variously acclimatized groups of fish. The straight lines are regression curves calculated from the original data on individual fish using the method of least squares. The equations for these curves are:

$$Cl_s = 211{\cdot}9\,\Delta + 13{\cdot}4$$
$$Na_s = 234{\cdot}6\,\Delta + 14{\cdot}7$$

Chloride concentrations are represented by open symbols, sodium concentrations by solid symbols. Triangles represent experiments at 10° C., circles those at 20° C. Only fish sampled between September and April are included.

Muscle concentrations

The patterns developed for all muscle concentrations were very similar to that described for Δ. The data are presented in Tables 2, 3, 5 and 6. Fig. 3 shows the degree of correlation between muscle total solids and Δ.

There were several quantitative differences in comparison with serum concentrations. The differences which seem worthy of mention are:

(1) The 1- to 3-day stabilization level in half sea water was only 11 % above fresh-water control (18 % for serum concentration after three days), and concentration after 20 hr. in full sea water was only 33 % above control (56 % for serum concentration).

(2) There was a complete return to fresh-water control level after ten days in half sea water (serum concentration still 6 % above control at this time).

(3) There were no significant increases in Cl_m above fresh-water control in fish transferred to half sea water. The means are identical with control from 72 hr. onwards in half sea water on.

(4) Na_m showed a significant seasonal variation; Na_s did not.

Table 6. *Changes in serum and muscle potassium following application of osmotic stresses*

(September–April.)

State of acclimatization	K_s (m-equiv./l.) [$\bar{x}\pm$s.e. (N)]	K_m (m-equiv./kg.) [$\bar{x}\pm$s.e. (N)]
20° C. experiments		
FW, 0 hr.	4·2±0·6 (9)	142·6±1·2 (12)
FW, 72–240 hr.	5·1±0·5 (13)	142·5±1·7 (14)
½ SW, 24 hr.	4·2±0·7 (9)	153·7±1·5 (25)
½ SW, 72 hr.	5·3±0·8 (9)	
½ SW, 240 hr.	3·8±0·7 (11)	141·8±1·0 (12)
SW, 21–30 hr.	6·8±0·6 (11)	180·8±1·2 (15)
½ SW, 240 hr., SW, 47 hr.	12·4±3·2 (5)	157·6±2·2 (5)
½ SW, 240 hr., SW, 240 hr.	4·0±0·4 (6)	151·7±6·2 (6)
10° C. experiments		
Hatchery controls	2·4±0·3 (12)	138·2±1·4 (12)
FW, 0 hr.	1·6±0·5 (6)	137·2±1·2 (6)
FW, 72–240 hr.	2·8±0·5 (11)	136·8±2·5 (6)
SW, 24–72 hr.	6·3±0·8 (11)	156·4±2·3 (12)
SW, 240 hr.	4·3, 5·2 (2)	132, 156 (2)

Table 7. *Changes in serum total phosphorus following application of osmotic stresses*

(September–April.)

State of acclimatization	P (mg./l.) [$\bar{x}\pm$s.e. (N)]
20° C. experiments—breeding season	
FW, 0 hr.	120±11 (4)
FW, 72–240 hr.	70±4 (7)
½ SW, 24–72 hr.	72±3 (13)
½ SW, 240 hr.	101±9 (7)
SW, 21–30 hr.	128±23 (6)
½ SW, 240 hr., SW, 47 hr.	159±38 (5)
½ SW, 240 hr., SW, 240 hr.	100±12 (6)
20° C. experiments—spring	
FW, 0 hr.	131±11 (5)
FW, 72–240 hr.	110±7 (6)
½ SW, 24–72 hr.	116±7 (12)
½ SW, 240 hr.	120±2 (4)
SW, 21–30 hr.	178±32 (5)
10° C. experiments—spring unless noted	
Hatchery controls	118±2 (6)
Hatchery controls, breeding season	81±4 (6)
FW, 0 hr.	150±14 (6)
FW, 72–240 hr.	109±8 (11)
SW, 24–72 hr.	150±12 (12)
SW, 240 hr.	95, 40 (2)

A final point is that the data on Cl_s and Cl_m considered together indicate that the volume of extracellular fluid in the muscles is quite independent of the state of salinity acclimatization of the fish. Assuming that all muscle chloride is extracellular, the ratio Cl_m/Cl_s (cf. last column in Table 3) provides an estimate of this quantity. There are no statistically significant variations in this ratio (the figures in parentheses in the last column of Table 3 are approximate 95% confidence intervals for the ratios listed, calculated from mean $Cl_m \pm 2$ S.E.).

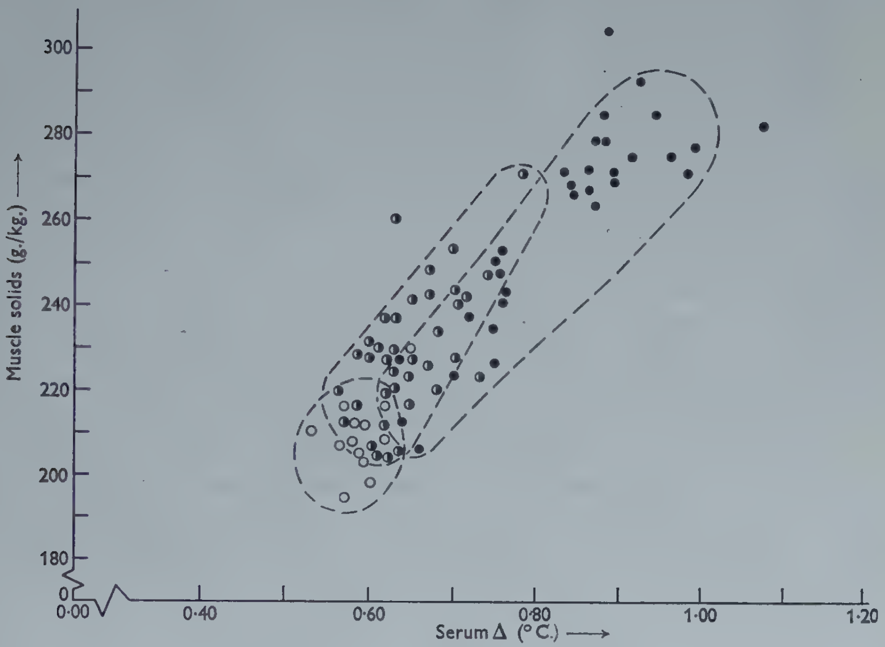


Fig. 3. Relations between muscle solids concentration and serum Δ in Massachusetts fish transferred from fresh water to half-strength and to full-strength sea water at 20° C. between September and April. Dashed lines roughly indicate boundaries of groups.

Experiments at 20° C., June to August

The experiments carried out at 20° C. between June and August were not as complete as those between September and April, but they serve to show that the brown trout in summer is a very different fish, at least with regard to osmotic and ionic regulatory abilities, from the brown trout at other times of year.

Two groups of fresh-water (hatchery) controls were sampled in late June and mid-August. These two series were insignificantly different from one another with respect to Cl_s and total muscle solids (the only analyses done on the June series). The August fish showed significant changes in all serum and muscle concentrations that were invariant between September and April—see Table 8.

The data on variations in total muscle solids following acclimatizations to half-strength and to full-strength sea water during the summer were as follows (figures are means ± 1 S.E.): Fresh water: 217·5 ± 2·8 (N = 11); half sea water, 24–29 hr.:

Table 8. *Seasonal variability in fresh-water control fish*

(20±2° C.)

Concentration	September–April [$\bar{x}$±s.e. (N)]	August [$\bar{x}$±s.e. (N)]
Δ (° C.)	0·587±0·008 (14)	0·553±0·009 (6)
Cl_s (m-equiv./l.)	140·5±1·1 (14)	123·2±2·0 (6)
Na_s (m-equiv./l.)	149·3±1·4 (13)	142·2±2·7 (6)
K_s (m-equiv./l.)	5·1±0·5 (13)	3·5±0·3 (6)
P (mg./l.)	70 (Sept.); 110 (April)	165± 10 (6)
Muscle solids (g./kg.)	209·7±2·3 (14)	217·2±3·0 (6)
Cl_m (m-equiv./kg.)	9·6±0·6 (14)	11·3±0·9 (6)
Na_m (m-equiv./kg.)	18·8 (Sept.); 12·0 (April)	10·1±0·6 (6)
K_m (m-equiv./kg.)	142·5±1·7 (14)	129·3±1·5 (6)

259·4±4·7 (N = 5); half sea water, 240 hr.: 234·4±5·3 (N = 5); sea water, 12–16 hr.: 297·0±3·2 (N = 5); half sea water, 240 hr., sea water, 240 hr.: 238·0±7·0 (N = 3).

Comparison of these data with Table 2 shows that in all states of acclimatization brown trout in summer were more concentrated (by 7–32 g./kg.) than brown trout during the rest of the year. It can also be seen that summer fish were much poorer regulators than fish during the rest of the year. Long-term acclimatizations to half, or to half and then to full, sea water did not result in as complete returns toward fresh-water levels as occurred between September and April. Furthermore, fish transferred from fresh water to full sea water survived for only about 12–16 hr. (compared to 20–30 hr. during the rest of the year), and became more concentrated. It appears that the brown trout decreases markedly in osmoregulatory ability in the months prior to, but seemingly not during, the breeding season.

Experiments at 10° C., *February*

A lowering of the temperature by 10° C. resulted in a marked improvement in the resistance of brown trout to sudden osmotic stresses. As regards survival, all fish were able to survive direct transfer from fresh water to full sea water for at least 2 days. About one-fifth of the number so transferred and not used at earlier times were able to survive this transition indefinitely (at least for 10 days).

The general pattern of concentration changes at 10° C. was similar to that occurring at 20° C., but with quantitative differences. The fresh-water controls showed the same lack of effect of handling and starvation noted for the 20° C. experiments (all differences in means insignificant at the 1 % level), but Δ and Cl_s were 5 % lower than in fish kept at 20° C. (difference statistically significant at the 1 % level). K_s in fresh water was about 2 m-equiv./l. lower at 10° C. than at 20° C. (also significant at the 1 % level).

The stabilization level reached by Δ after one day in full sea water was 35 % above fresh-water control (compared to 56 % at 20° C.). Total muscle solids were only 20 % above control at the same time (compared to 33 % at 20° C.). The two living fish sampled after 10 days in full sea water were almost as different from one another as the most extreme fish in the group which had spent 10 days in full sea water at 20° C.

The pattern shown by Δ is presented in Fig. 4. The average curve for the full sea water transfers at 20° C. is included in this figure for reference.

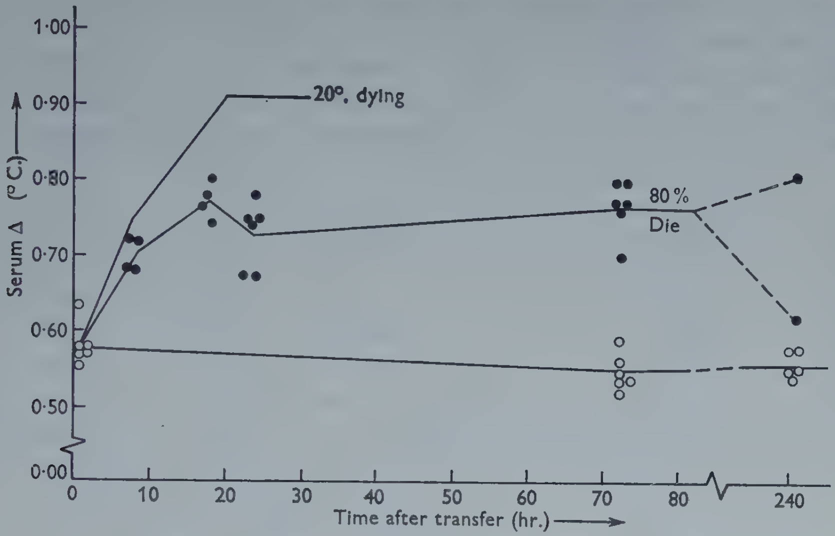


Fig. 4. Time course of changes in serum Δ in Massachusetts hatchery fish following transfers from fresh water to full-strength sea water at 10° C. in February.

The preliminary experiment using fish from the Connecticut hatchery was also done at 10° C. in February. Transfers were from fresh water to sea water of only 28‰ salinity. The general pattern of concentration changes that occurred was the same as for the Massachusetts fish, but blood concentrations in fresh water were significantly higher (Δ = 0·615 ± 0·015° C. (N = 4)), rates of increase of internal concentration following transfer were somewhat lower (they were fish in the same size range), and 1- to 3-day stabilization levels were higher (Δ = 0·794 ± 0·019° C. (N = 12)) even though the external concentration was lower.

The overall pattern of regulation of total serum concentration in Massachusetts *Salmo trutta* between September and April is summarized in the classic form of a plot of total internal *v.* total external concentration in Fig. 5. The variability of the fish acclimatized to full sea water gradually and for a long period at 20° C. (perhaps also at 10° C.) was probably an experimental artefact.

DISCUSSION

The data described above permit a description of osmotic and ionic regulation in the brown trout that is, in some ways, more detailed than for any other fish.

Seasonal effects

Seasonal changes, especially the onset of the breeding season, may or may not be associated with changes in the relation between internal and external media.

In female eels (Fontaine & Koch, 1950, for summary) overall osmoregulatory ability is apparently little affected at this period. Sticklebacks (*Gasterosteus* and *Pungitius*), however, show less osmotic regulation and are less euryhaline (Heuts, 1942, 1943, 1945; Koch & Heuts, 1942, 1943). The seasonal pattern in the brown trout is somewhat different.

D. C. W. Smith (1956) studied seasonal changes in tolerance of brown trout to artificially increased salinities (increased by addition of sodium chloride) above a previously determined 'physiological maximum' (only 24‰!). Results in two successive years differed by over 100% in absolute values, but showed more or less consistent patterns of seasonal differences. Salinity tolerance was lowest during the summer, went through a peak, was low again from October to December (the breeding season), then went through a broad peak during the spring.

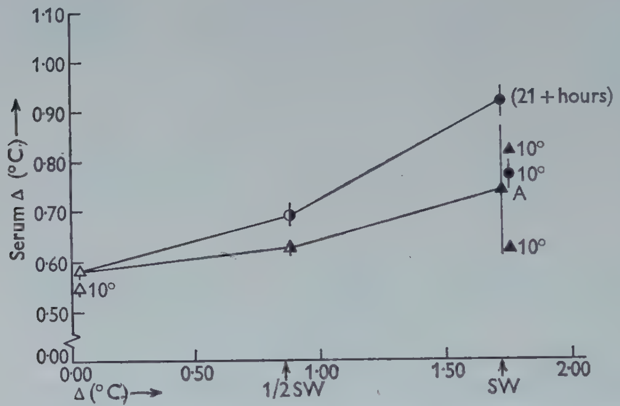


Fig. 5. Relations between freezing-point depressions of serum and external environment in Massachusetts hatchery fish acclimatized to half-strength and to full-strength sea water for varying periods at 20° C. and 10° C. Triangles indicate long-term acclimatizations (240 or more hours), circles short-term acclimatizations (72 hours unless noted otherwise). *A* means acclimatized to half-strength sea water for 240 hours first. 10° C. points are noted as such. The two 10° C. triangles in full-strength sea water are for individual fish. All other symbols indicate means for groups ± two standard errors.

The results here reported agree with Smith's as regards the summer decrease in viability, and show in addition that actual decreases in regulatory abilities are involved; the breeding season, however, was not associated with any changes in these abilities. The only changes correlated with this latter period were in serum phosphorus and total muscle sodium concentrations. Survival and regulatory abilities were seemingly constant over the entire period from autumn to spring.

Reasons for this rather odd pattern of changes in regulatory abilities are not readily apparent. Two environmental correlations which might have affected endocrine functions in some way seem possible, however:

(1) Regulatory abilities may be influenced by photoperiod. The experimental series between September and April were carried out during those parts of the year with nights longer than days. The reverse was true for the summer series.

(2) Regulatory abilities may be influenced by the long-term thermal history of the fish. The September to April series involved fish acclimatized for periods of weeks to hatchery water temperatures in the range 2–14° C. The summer series involved fish acclimatized to temperatures of 18–24° C.

The seasonal changes in muscle sodium are unaccounted for. Serum phosphorus was the only other concentration studied which varied significantly seasonally outside the summer. Its variations are probably due to various seasonal metabolic changes (Ichikawa, 1955; Laskowski, 1936).

Serum phosphorus concentrations during the breeding season were 20–50 mg./l. below the levels measured between February and April and about 90 mg./l. below summer levels. The figures given by Laskowski (1936) for one brown trout during the breeding season and one brown trout outside the breeding season show the opposite effect—breeding is approximately twice non-breeding. The reason for this discrepancy is unknown.

The fact that brown trout in fresh water in summer have different serum ion concentrations from brown trout in fresh water in winter is probably the major reason for the discrepancies existing between serum sodium and potassium concentrations reported for this species by Spalding (in Jones, 1956) and by Phillips & Brockway (1958). Spalding obtained her samples during the winter, Phillips & Brockway obtained theirs during the summer

Water and ion movements between muscles and blood

(*a*) *Water movements*

Studies of total muscle concentration (g. solids/kg. muscle wet weight or Δ of expressed tissue fluid) indicate that this quantity usually varies in a similar fashion to total blood concentration—the muscles are in some sort of osmotic equilibrium with the blood (in salmonids: Busnel, 1942; Greene, 1926; *Anguilla*: Callamand, 1943; Drilhon, 1937, 1943, Koch, 1949; Callamand *et al.* (1951), also working with eels, did not find such parallelism). This is probably true in the brown trout as well. The plot of total muscle concentration against serum Δ for the present experiments at 20° C. shown in Fig. 3 is not incompatible with the view that the muscles are in osmotic equilibrium with the blood (whether or not isotonic is another question—cf. Brodsky, Appleboom, Dennis, Rehm, Miley & Diamond, 1957).

A plot of total muscle concentration *v.* serum Δ for fish transferred to full sea water at 10° C. and 20° C. shows the same relation.

The fact that most of the changes in total muscle solids are due to changes in intracellular water content may also be demonstrated.

Table 3 shows that the extracellular volume in the muscles is small and constant. Changes in water content of the muscles occurred under some conditions which varied from 20–70 g./kg. (Table 2). With constant extracellular volume, this water could have come from no place but the fibres. Even if the statistically insignificant fluctuations in chloride space indicated in Table 3 are real they are insufficient to account for the total water-content changes which occurred.

The data of Callamand *et al.* (1951) on muscle chloride concentrations in hypophysectomized and intact female eels transferred from fresh water to full sea water indicate that a similar situation exists in *Anguilla*.

(*b*) *Ion movements*

A consideration of all the data obtained on ion concentrations in serum and muscle permits a critical test of Drilhon's (1937) hypothesis that the muscles of a fish can act as storage sites for ions.

We have seen that extracellular volume in the muscles of the brown trout is small and quite constant, at least between September and April. Serum sodium and potassium concentrations for these same fish are known, also total muscle sodium and potassium concentrations. Combining these data with those on total muscle solids (hence total muscle water), intracellular concentrations of these two ions can be calculated from the equation:

$$[A]_i = \frac{A_t - [A]_s\, V_e}{V_t - V_e}.$$

$[A]_i$ = intracellular concentration of ion species A (m-equiv./kg. intracellular water). A_t = total quantity of A (m-equiv.) in 1 kg. of wet muscle. $[A]_s$ = serum concentration of A (use m-equiv./l. as approximation to m-equiv./kg. water). V_t = total weight of water (kg.) in 1 kg. of wet muscle. V_e = weight of extracellular water (kg.), assumed to be present as serum, in 1 kg. of wet muscle.

The results of such calculations for muscle sodium in variously acclimatized brown trout are presented in Table 9. Comparable data for potassium may be taken directly from Table 6, since the extracellular space is small and serum potassium concentrations are low. The muscle sodium data will be considered first.

It should be pointed out that indistinct end-points in the muscle chloride analyses resulted in low precision for the extracellular volume estimations. Small changes in calculated extracellular volume produce large changes in intracellular sodium concentration. By reason of these analytical errors the amount of sodium calculated to be present extracellularly sometimes exceeds the amount of sodium measured in the muscle. Such cases are listed in Table 9 as having zero intracellular sodium. It seems probable that their true intracellular sodium concentrations were low.

Approximate 90% confidence intervals for intracellular sodium concentrations are given in parentheses after the mean values in Table 9. These intervals were calculated in the same manner as the means, but using the following figures. Upper bound: Mean total muscle sodium + 2 S.E., extracellular volume from mean muscle chloride − 2 S.E. Lower bound: Mean total muscle sodium − 2 S.E., extracellular volume from mean muscle chloride + 2 S.E.

Despite the uncertainty just mentioned, the calculated intracellular sodium concentrations bring up several items of interest.

The major point is that, with the exception of fish transferred directly from fresh water to full sea water at 20° C., both during and outside the breeding season,

Table 9. *Changes in intracellular sodium in the muscles following application of osmotic stresses*

(September–April.)

State of acclimatization	Na$_i$ (m-equiv./kg. cell water)
20° C. experiments—breeding season	
FW, 0 hr.	6·7 (2–12)*
FW, 72–240 hr.	12·1 (7–17)
½ SW, 24 hr.	6·4 (0–13)
½ SW, 72 hr.	4·5 (0–11)
½ SW, 240 hr.	10·6 (7–14)
SW, 21–30 hr.	16·3 (7–25)
½ SW, 240 hr.–SW, 47 hr.	6·0 (0–15)
½ SW, 240 hr.–SW, 240 hr.	0 (0–8)
20° C. experiments—spring	
FW, 0 hr.	0 (0)
FW, 72–240 hr.	2·6 (0–8)
½ SW, 24 hr.	0 (0–5)
½ SW, 72 hr.	2·4 (0–6)
½ SW, 240 hr.	0 (0–3)
SW, 21–30 hr.	14·0 (4–24)
10° C. experiments—spring unless noted	
Hatchery controls	0 (0–1)
Hatchery controls, breeding season	2·9 (0–7)
FW, 0 hr.	0·8 (0–4)
FW, 72 hr.	0 (0–8)
SW, 24 hr.	3·0 (0–8)
SW, 72 hr.	3·0 (0–8)
SW, 240 hr.	0, 0

* Approximate 90 % confidence interval. (See p. 246.)

intracellular sodium concentrations during each experimental series were fairly constant. This argues that little, if any, sodium invaded the fibres even during the periods when they were considerably dehydrated and serum concentrations were high. If anything, the fibres excreted sodium during periods of dehydration. Thus, the muscles of the brown trout almost certainly did not act as storage sites for sodium during or after adjustments to non-fatal osmotic stresses.

The data on fish acclimatized to full sea water at 20° C. bring up a point which may bear on the question of why some fish are euryhaline, others not. Many of the fish transferred directly from fresh water to full sea water showed considerable increases in intracellular sodium, these changes being clearest outside the breeding season. But fish acclimatized to half sea water first, while in some cases having serum concentrations as high as those in the direct transfers, seemingly were able to maintain low intracellular sodium concentrations. Perhaps this apparent increase in the ability of acclimatized fish to prevent the invasion of their muscle fibres by large amounts of sodium is one of the factors which enabled them to survive blood concentrations equal to those associated with mortality in the direct transfers.

Stenohaline fishes seem to be unable to keep sodium out of their muscle fibres, even with long-term acclimatizations (Kaplanski & Boldirewa, 1933; Drilhon, 1937). Steinbach (1954) and others (cf. Glynn, 1957, p. 296) have pointed out the inhibitory effects which low concentrations of sodium have on various enzyme systems important in muscle metabolism. It is tempting to speculate that differences in ability to prevent sodium invasion of muscle fibres are a determining factor in the degree of euryhalinity shown by fishes, and that the mechanism of the effect is that discussed by Steinbach.

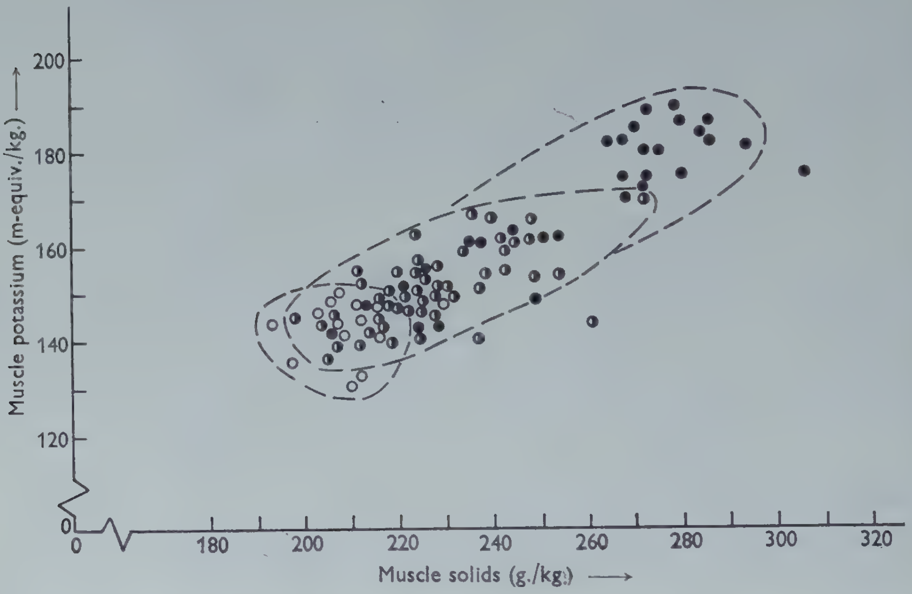


Fig. 6. Relations between muscle potassium, K_m, and muscle solids concentration in Massachusetts hatchery fish transferred from fresh water to half-strength and to full-strength sea water at 20° C. between September and April. Dashed lines roughly indicate boundaries of the three groups.

That temperature, as well as acclimatization time, may be significant in keeping sodium out of muscle fibres is illustrated by the fish transferred directly from fresh water to full sea water at 10° C. and 20° C. in the spring. The 20° C. fish, as noted, considerably increased their intracellular sodium concentrations—the 10° C. fish seemingly did not. Serum concentrations at 10° C. never reached the high levels noted at 20° C., which in itself may explain the greater survival noted at 10° C. However, the difference in behaviour of muscle sodium concentration may also have been significant.

The potassium data indicate that the muscles did not act as reservoirs for this ion, either. Small differences in large quantities are involved here, however, so it is difficult to be certain. The close correlation existing between muscle potassium and total muscle concentrations is shown in Fig. 6.

Ecological implications

Gordon (1959) has shown that responses of Scottish brown and sea trout to osmotic stresses are similar to those described for Massachusetts fish in the present paper. The many generations of genetic isolation that separate European and American populations of *Salmo trutta* have resulted in only quantitative differences in osmotic and ionic regulatory mechanisms.

In the same regard it is interesting that serum Na:Cl ratios in all groups of American fish are in the range 1·06–1·13, which is the range found in Scottish fresh water *S. trutta*. Scottish sea trout have serum Na:Cl ratios in the range 1·18–1·28. Perhaps this indicates that at least the great majority of the ancestors of present-day American *S. trutta* were brown trout, only a few, if any, having been sea trout.

Finally, the experiment at 10° C. seems to present an interesting possibility for explaining a zoogeographic problem in the distribution of the anadromous habit in at least some fishes. Brook trout (*Salvelinus fontinalis*), lake whitefish (*Coregonus clupeaformis*), various ciscoes (*Coregonus* (*Leucichthys*) *spp.*), and many other fishes are commonly anadromous in the far northern parts of their ranges (e.g. Hudson Bay) but seldom or never so near their more southern limits (Walters, 1955; Wohlschlag, 1957). Assuming that these fish make their transitions from fresh water to salt water fairly rapidly, one might postulate that, at the higher temperatures encountered in the southern parts of their ranges, they are unable to maintain their blood and muscle concentrations on a level compatible with survival. At lower temperatures in the more northerly parts of their ranges they can maintain lower blood and muscle concentrations and thus survive.

There would seem to be considerable adaptive advantage in going to sea, primarily from the standpoint of increased food supply. Some counter-selection is therefore probably operating to prevent such behaviour in the southern parts of the ranges of these fish. Perhaps the temperature effect on short-term levels of regulation of blood and muscle concentrations is part of this counter-selection.

SUMMARY

1. The osmotic and ionic regulatory abilities of adults of the euryhaline brown trout (*Salmo trutta*) have been studied in experiments roughly duplicating the stresses of migration from fresh water to the sea. Brown trout will survive indefinitely in full sea water if acclimatized to it at rates inversely related to the temperature.

2. Blood serum samples have been analysed for Na, K, Cl, total P and Δ; muscle samples for Na, K, Cl and total solids. Changes in the concentrations of these constituents following transfers from fresh water to 50% and 100% sea water have been studied. Transfers were made throughout the year and at temperatures of 10° C. and 20° C.

3. Following transfers to 50% sea water at 20° C. blood concentrations rose

significantly above fresh-water levels, but returned very nearly to these levels after about a week. Transfer from 50% sea water to 100% sea water at 20° C. caused the same sequence of changes. Transfer to 100% sea water at 20° C. was fatal, and associated with very high serum concentrations. Many fish survived transfer to 100% sea water at 10° C., however, and showed evidence of internal concentrations returning to fresh-water levels after 10 days. The brown trout is strongly homoiosmotic on a long-term basis.

4. Both survival and regulatory ability were lower during the summer. There were also seasonal variations in the blood and muscle concentrations of fish in a given state of acclimatization.

5. Muscle concentration changes closely paralleled blood changes. Extracellular volume remained constant, so muscle concentration changes were attributable to changes in intracellular water. The muscles did not act as storage sites for sodium and potassium.

These studies have been supported by National Science Foundation predoctoral fellowships for 1954–1957, a Fulbright Fellowship to the United Kingdom in 1957–1958 and a grant from the Higgins Fluid Research Fund, Yale University. This paper is a portion of a dissertation presented to the faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy. (Contribution number 989 from the Woods Hole Oceanographic Institution.)

The aid and advice of many people have been gratefully accepted in the course of this work. I would like to express my appreciation to them all, especially the following: Drs E. J. Boell, T. H. Waterman, G. E. Pickford and L. M. Passano, members of my Ph.D. dissertation committee at Yale University; Drs C. O'D. Iselin, B. H. Ketchum and A. C. Redfield, for permitting use of the excellent facilities of the Woods Hole Oceanographic Institution; MM. J. Galligan and D. Moss, Connecticut State Board of Fisheries and Game, MM. R. Bitzer and J. Macomber, Massachusetts State Division of Fisheries and Game, and Mr H. Robbins, Robbins Fish Hatchery, Wareham, Mass., for supplying fish; Drs C. M. Breder, Jr, D. Merriman, J. A. Ramsay, J. Ryther and H. Werntz, and Miss P. Rasquin, for helpful advice and discussion. Miss C. Luhrs very kindly typed the first draft of the manuscript.

REFERENCES

BEADLE, L. C. (1957). Comparative physiology: Osmotic and ionic regulation in aquatic animals. *Ann. Rev. Physiol.* **19**, 329–58.

BENDITT, E., MORRISON, P. & IRVING, L. (1941). The blood of the Atlantic salmon during migration. *Biol. Bull., Woods Hole*, **80**, 429–40.

BERRY, J. W., CHAPPELL, D. G. & BARNES, R. B. (1946). Improved method of flame photometry. *Industr. Engng Chem.* (*Anal. Ed.*), **18**, 19–24.

BLACK, V. S. (1957). Excretion and osmoregulation. In *The Physiology of Fishes* (ed. Brown), vol. 1, 163–205.

BOUCHER-FIRLY, S. (1935). Recherches biochimiques sur les téléostéens apodes (Anguille, Congre, Murène). *Ann. Inst. Océanogr. Monaco*, **15**, 217–327.

BOYLE, P. J. & CONWAY, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1–63.

BRODSKY, W. A., APPLEBOOM, J. W., DENNIS, W. H., REHM, W. S., MILEY, J. F. & DIAMOND, I. (1956). The freezing point depression of mammalian tissues in relation to the question of osmotic activity of cell fluid. *J. Gen. Physiol.* **40**, 183–99.

BURDEN, C. E. (1956). The failure of hypophysectomized *Fundulus heteroclitus* to survive in fresh water. *Biol. Bull., Woods Hole*, **110**, 8–28.

BUSNEL, R. G. (1942). Recherches de physiologie appliquées à la pisciculture: à propos de la migration de la truite arc-en-ciel. *Bull. Français Pêche et Pisciculture*, **15**, no. 127, 45–65.

CALLAMAND, O. (1943). L'Anguille Européenne (*Anguilla anguilla* L.). Les bases physiologiques de sa migration. *Ann. Inst. Océanogr. Monaco*, **21**, 361–440.

CALLAMAND, O., FONTAINE, M., OLIVEREAU, M. and RAFFY, A. (1951). Hypophyse et osmorégulation chez les poissons. *Bull. Inst. Océanogr., Monaco*, no. 984, 1–7.

CHEN, P. S., JR, TORIBARA, T. Y. & WARNER, H. (1956). Microdetermination of phosphorus. *Anal. Chem.* **28**, 1756–7.

CONWAY, E. J. (1935). An absorption apparatus for the micro-determination of certain volatile substances. III. The micro-determination of chloride, with application to blood, urine and tissues. *Biochem. J.* **29**, 2221–35.

CONWAY, E. J. (1957). Nature and significance of concentration relations of potassium and sodium ions in skeletal muscle. *Physiol. Rev.* **37**, 84–132.

DEKHUYZEN, M. C. (1905). Sur la pression osmotique dans le sang et dans l'urine des poissons. *Arch. néerl. Sci.* **10**, 121–36.

DRILHON, A. (1937). Étude des échanges minéraux chez les poissons homéiosmotiques. *C.R. Acad. Sci. Paris*, **204**, 1502–3.

DRILHON, A. (1943). Métaux alcalins et alcalino-terreux chez les téléostéens apodes. *C.R. Soc. Biol. Paris*, **137**, 300–2.

DUVAL, M. (1925). Recherches sur le milieu intérieur des animaux aquatiques. Modifications sous l'influence du milieu extérieur. *Ann. Inst. Océanogr. Monaco*, **2**, 233–407.

FONTAINE, M. (1930). Modifications du milieu intérieur des poissons potamotoques au cours de la reproduction. *C.R. Acad. Sci. Paris*, **191**, 736–7.

FONTAINE, M. (1954). Du déterminisme physiologique des migrations. *Biol. Rev.* **29**, 390–418.

FONTAINE, M. (1956). The hormonal control of water and salt-electrolyte metabolism in fish. *Mem. Endocrin.* no. 5, 69–82.

FONTAINE, M., CALLAMAND, O. & VIBERT, R. (1950). La physiologie du saumon. *Ann. Sta. Cent. Hydrobiol. App.* **3**, 15–26.

FONTAINE, M. & FIRLY, S. (1932). Influence des changements de salinité sur la teneur en phosphore minéral du sérum des poissons. *C.R. Soc. Biol. Paris*, **109**, 1271–3.

FONTAINE, M. & KOCH, H. J. (1950). Les variations d'euryhalinité et d'osmorégulation chez les poissons. *J. Physiol. Path. Gén.*, **42**, 287–318.

FORSTER, R. P. (1953). A comparative study of renal function in marine teleosts. *J. Cell. Comp. Physiol.* **42**, 487–509.

GARRARD, B. M. (1935). The osmotic pressure of the blood of *Fundulus* living in salt water and in fresh water. *J. Physiol.* **85**, 6*P*.

GLYNN, I. M. (1957). The ionic permeability of the red cell membrane. *Progr. Biophys.* **8**, 241–307.

GORDON, M. S. (1957). Observations on osmoregulation in the Arctic char (*Salvelinus alpinus* L.). *Biol. Bull., Woods Hole*, **112**, 28–33.

GORDON, M. S. (1959). Osmotic and ionic regulation in Scottish brown and sea trout (*Salmo trutta* L.). *J. Exp. Biol.* (in the Press).

GRAFFLIN, A. L. (1935). Chloride and total osmotic pressure in the blood of marine teleosts. *Biol. Bull., Woods Hole*, **69**, 245–58.

GREENE, C. W. (1904). Physiological studies of the chinook salmon. *Bull. U.S. Bur. Fish.* **24**, 429–56.

GREENE, C. W. (1926). The physiology of the spawning migration. *Physiol. Rev.* **6**, 201–41.

GUEYLARD, F. (1924). De l'adaptation aux changements de salinité. Recherches biologiques et physico-chimiques sur l'Épinoche (*Gasterosteus leiurus* Cuv. et Val.). *Arch. Phys. biol.* **3**, 79–197.

HEILBRUNN, L. V. & HAMILTON, P. G. (1942). The presence of chloride in muscle fibers. *Physiol. Zoöl.* **15**, 363–74.

HEUTS, M. J. (1942). Chloride excretie bij *Gasterosteus aculeatus* L. *Ann. Soc. zool. Belg.* **73**, 69–72.

HEUTS, M. J. (1943). La régulation osmotique chez l'épinochette (*Pygosteus pungitius* L.). *Ann. Soc. zoöl. Belg.* **74**, 99–105.

HEUTS, M. J. (1945). La régulation minérale en fonction de la température chez *Gasterosteus aculeatus*. Son importance au point de vue de la zoogéographie de l'espèce. *Ann. Soc. zool. Belg.* **76**, 88–99.

HOAR, W. S. (1953). Control and timing of fish migration. *Biol. Rev.* **28**, 437–52.

HOAR, W. S., BLACK, V. S. & BLACK, E. C. (1951). Some aspects of the physiology of fish. *Publ. Ontario Fish. Res. Lab.* **71**. *Univ. Toronto Biol. Ser.* no. 59, 1–111.

ICHIKAWA, R. (1955). Seasonal difference in the amount of inorganic phosphorus in blood serum of fishes. *Rec. Oceanogr. Works, Japan*, **2**, 113–16.

JONES, I. C. (1956). The role of the adrenal cortex in the control of water and salt-electrolyte metabolism in vertebrates. *Mem. Endocrin.* no. 5, 102–20.

KAPLANSKI, S. & BOLDIREWA, N. (1933). Über den Mineralstoffgehalt des Muskelgewebes von Süsswasserfischen bei gesteigertes Konzentration der Mineralsalze im Wasser. *Biochem. Z.* **265**, 422–5.

KINNE, O. (1952). Ein neues Gerät zur Bestimmung der Gefrierpunktserniedrigung kleiner Flüssigkeitsmengen. *Veröff. Inst. Meeresforsch. Bremerhaven*, **1**, 47–51.

KOCH, H. J. (1949). Quelques caractéristiques osmotiques de l'Anguille femelle jaune et argentée. *Arch. int. Physiol.* **57**, 125–32.

KOCH, H. J. & HEUTS, M. J. (1942). Influence de l'hormone thyroïdienne sur la régulation osmotique chez *Gasterosteus aculeatus* L. forme *gymnurus* Cuv. *Ann. Soc. zool. Belg.* **73**, 165–72.

KOCH, H. J. & HEUTS, M. J. (1943). Régulation osmotique, cycle sexuel et migration de reproduction chez les épinoches. *Arch. int. Physiol.* **53**, 253–66.

KUBO, T. (1953). On the blood of salmonid fishes of Japan during migration. I. Freezing point of blood. *Bull. Fac. Fish. Hokkaido Univ.* **4**, 138–48 (in Japanese, with English summary).

LASKOWSKI, M. (1936). Über das Vorkommen des Serumvitellins im Blute der Wirbeltiere. *Biochem. Z.* **284**, 318–21.

MARGOSHES, M. & VALLEE, B. L. (1956). Instrumentation and principles of flame spectrometry. Effect of extraneous ions in simultaneous determination of five elements. *Anal. Chem.* **28**, 180–4.

MULLINS, L. J. (1950). Osmotic regulation in fish as studied with radio-isotopes. *Acta physiol. scand.* **21**, 303–14.

PARKS, T. S., JOHNSON, H. O. & LYKKEN, L. (1948). Errors in the use of a model 18 Perkin–Elmer flame photometer for the determination of alkali metals. Interferences of common metals, acids and solvents. *Anal. Chem.* **20**, 822–5.

PARRY, G. (1958). Size and osmoregulation in salmonoid fishes. *Nature, Lond.*, **181**, 1218–19.

PHILLIPS, A. M., JR, & BROCKWAY, D. R. (1958). The inorganic composition of brown trout blood. *Progr. Fish Cult.* **20**, 58–61.

PICKFORD, G. E. & ATZ, J. W. (1957). *The Physiology of the Pituitary Gland of Fishes*, 613 pp. *N.Y. Zool. Soc.*

RAMSAY, J. A., BROWN, R. H. J. & FALLOON, S. W. H. W. (1953). Simultaneous determination of sodium and potassium in small volumes of fluid by flame photometry. *J. Exp. Biol.* **30**, 1–17.

SHAW, F. H., SIMON, S. E. & JOHNSTONE, B. M. (1956). The non-correlation of bioelectric potentials with ionic gradients. *J. Gen. Physiol.* **40**, 1–17.

SHAW, F. H., SIMON, S. E., JOHNSTONE, M. B. & HOLMAN, M. E. (1956). The effect of changes of environment on the electrical and ionic pattern of muscle. *J. Gen. Physiol.* **40**, 263–88.

SHENK, W. D. (1954). Tissue chloride. *Arch. Biochem. Biophys.* **49**, 138–48.

SIMON, S. E., SHAW, F. H., BENNETT, S. & MULLER, M. (1957). The relationship between sodium, potassium and chloride in amphibian muscle. *J. Gen. Physiol.* **40**, 753–77.

SMITH, D. C. W. (1956). The role of the endocrine organs in the salinity tolerance of trout. *Mem. Endocrin.* no. 5, 83–101.

SMITH, H. W. (1930). The absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.* **93**, 480–505.

STEINBACH, H. B. (1954). The regulation of sodium and potassium in muscle fibers. *Symp. Soc. Exp. Biol.* **8**, 438–52.

STEPHENSON, W. K. (1957). Membrane potential changes and ion movements in the frog sartorius muscle. *J. Cell. Comp. Physiol.* **50**, 105–28.

VAN SLYKE, D. D. (1923). The determination of chlorides in blood and tissues. *J. Biol. Chem.* **58**, 523–9.

VON BUDDENBROCK, W. (1956). *Vergleichende Physiologie. III. Ernährung, Wasserhaushalt und Mineralhaushalt der Tiere*, 677 pp. Basel: Birkhauser.

WALTERS, V. (1955). Fishes of western Arctic America and eastern Arctic Siberia. Taxonomy and zoogeography. *Bull. Amer. Mus. Nat. Hist.* **106**, 255–368.

WERNTZ, H. O. (1957). Osmotic adaptations of species of *Gammarus* to changes in the external concentration. Unpublished Ph.D. thesis, Yale University.

WOHLSCHLAG, D. E. (1957). Differences in metabolic rates of migratory and resident freshwater forms of an arctic whitefish. *Ecology*, **38**, 502–10.

OSMOTIC AND IONIC REGULATION IN SCOTTISH BROWN TROUT AND SEA TROUT (*SALMO TRUTTA* L.)

By MALCOLM S. GORDON*

Department of Zoology, Yale University, and Woods Hole Oceanographic Institution†

(*Received* 11 *July* 1958)

The brown trout (*Salmo trutta* L.) was originally a Palearctic fish, widely distributed in Europe and western Asia from the Mediterranean to the Arctic. Many river drainage systems in this area contained, and still contain, two or even three more or less morphologically and behaviourally recognizable forms of this species. These forms are the 'lake trout', living in fresh-water lakes, the 'brown trout', living in fresh-water streams, and the 'sea trout', an anadromous form. The question of whether or not these forms, especially the brown trout and the sea trout, are 'really' (equals genetically) different from one another has been debated by fishermen and others for centuries.

Trewavas (1953) reviewed the most significant portions of the voluminous literature on this subject. She concluded that average differences in some characters (meristic characters, age at maturity and frequency of spawning) do separate some populations of the two types, but these do not separate all brown trout from all sea trout. Trewavas favoured the view that the forms are ecotypes of a single species, although there are difficulties attendant on the application of even this generalized term.

The results of hatchery breeding experiments carried out under fairly uniform conditions by Skrochowska (1951) were unknown to Trewavas at the time she wrote her paper, but confirm her view. This also is the case with tagging experiments reported by Skrochowska (1953), though these latter data are not as numerous as one might wish. Svärdson (1955) further supports Trewavas and presents an ingenious hypothesis explaining the existence of the two forms on the basis of responses of individual fish to environmental changes.

The morphological, behavioural and genetic evidence thus seems to indicate very strongly that brown trout and sea trout are at most morphotypes of a single species. Alm (1949), however, described morphological differences between the brown trout and the lake trout which seem to be genetically determined. Further, a very closely related form, the rainbow trout (*Salmo gairdneri*), shows what appear to be genetically determined differences in lateral line scale-counts between sea-run and fresh-water populations (Neave, 1944).

---

* Present address: Department of Zoology, University of California, Los Angeles 24, California.

† Contribution number 927 from the Woods Hole Oceanographic Institution.

That a polymorphic species differentiated into three more or less equally morphologically distinct forms, say *A*, *B* and *C*, would have *A* and *B* genetically different from one another, but *B* and *C* not so, would seem unlikely. It also seems doubtful that the rainbow trout is significantly older than the brown trout in an evolutionary sense. It would therefore be surprising if the brown trout, faced with environmental stresses identical to those faced by the rainbow trout, has not made genetic rearrangements similar to those made by the rainbow. One might also expect that such rearrangements would be reflected in the osmotic and ionic regulatory abilities of the fish.

During the course of a detailed study of osmotic and ionic regulation in an American hatchery population of *Salmo trutta* the writer observed several instances of variable behaviour between individual fish subjected to the same strong osmotic stress for long periods of time (Gordon, 1959). American brown trout are derived from European brown trout, and no one knows whether or not, or how many, European sea trout were included in the original *Salmo trutta* shipments sent to the United States. This fact, combined with the considerations mentioned above, made it seem reasonable to interpret the variability noted in the American fish as being a result of their being hybrid fish descended from physiologically distinct ancestors.

To test this hypothesis arrangements were made to obtain blood serum samples from known Scottish sea and brown trout subjected to osmotic stresses similar to those used on the American fish. The results of the analyses for freezing-point depression, chloride, sodium, and potassium that were carried out on these samples, the implications of these analyses for the brown trout or sea trout problem, and some comparisons of these data with material obtained from American fish form the basis for this paper.

MATERIALS AND METHODS

Known Scottish sea trout and brown trout were obtained from the area around Aberdeen between November 1956 and May 1957 by Dr Isabel W. Smith of the Marine Laboratory, Aberdeen. The fish were all sexually mature and varied in size from 18 to 55 cm. standard length. Temperature throughout was 10 ± 2° C. Fish were sampled as follows:

Sea trout. In November 1956 three sea trout were sampled after unknown, but perhaps quite short, periods in fresh water while on the upstream phase of their spawning migration. Two sea trout caught similarly were sampled 24 hr. after having been transferred to full sea water (salinity 32·6‰). Five more sea trout were sampled after having been maintained in full sea water for 5 months at the Marine Laboratory aquarium. In May 1957 seven sea trout were sampled after having been captured in commercial nets in an estuary containing water varying tidally in salinity from about 2–32‰.

Brown trout. In March 1957 a number of fish locally considered to be *Salmo levenensis*, or fresh-water brown trout, were removed from a reservoir near Aberdeen in which they had been stocked either 12 or 20 years earlier. Eleven of these were sampled while still in fresh water (some of them twice), then were transferred to one-half sea water (salinity 16‰) where they were kept for 10 days. Several of them were then sampled and all were transferred to full sea water. Additional samples were taken from varying numbers of these fish after 2 days in sea water, 10 days in sea water and 64 days in sea water (three survivors).

Blood samples were taken from the fish by Dr Smith by either heart or caudal artery puncture using a syringe with a fine needle. The samples were allowed to clot and the serum was pipetted off and placed in acid-cleaned vials. The vials were then tightly stoppered with paraffined corks, the samples frozen on dry ice and shipped in the frozen state by air express to the United States. Analyses for freezing-point depression (Δ), chloride (Cl), sodium (Na) and potassium (K) were then carried out by the author using methods described elsewhere (Gordon, 1959).

The multiple sampling of single brown trout in the present work is similar to the procedure used with Arctic char (*Salvelinus alpinus*) by Gordon (1957). It did not seem to cause any shock effects in the char.

Table 1. *Serum Δ in Scottish brown and sea trout*

State of acclimatization	N	Serum Δ (° C.) ($\bar{x}$ ± S.E.)
Brown trout		
F.W.	11	0·607 ± 0·011
½ S.W., 240 hr.	5	0·622 ± 0·038
½ S.W., 240 hr.–S.W., 48 hr.	4	0·748 ± 0·030
½ S.W., 240 hr.–S.W., 240 hr.	8	0·674 ± 0·021
½ S.W., 240 hr.–S.W., 64 days	3	0·637 ± 0·022
Sea trout		
F.W.	3	0·653 ± 0·045
S.W., 24 hr.	2	0·79, 0·81
S.W., 5 months	5	0·662 ± 0·014
Brackish, indefinite	7	0·666 ± 0·015

RESULTS

The data on serum Δ, Cl, Na and K in the sea and brown trout are presented in Tables 1–4. Gordon (1959) has shown that neither sex of the fish nor season (during the sampling periods involved here) have any significant effect on these concentrations.

The main point demonstrated by these data is that, with only one exception, the concentrations studied were insignificantly different (at the $P = 0{\cdot}05$ level by '*t*' test) in the brown trout and the sea trout when the fish were acclimatized to full sea water for long periods (10 days to 5 months). The one exception was serum Cl in brown trout acclimatized to sea water for 10 days as compared with sea trout in sea water for 5 months. These two means are highly significantly different ($P \ll 0{\cdot}01$). This exception is discussed below.

Similar agreement between the two forms occurred for their fresh-water concentrations as well, though the sample size of only two fish for most of the sea trout concentrations makes this point less certain.

Table 2. *Serum chloride in Scottish brown and sea trout*

State of acclimatization	N	Serum Cl (m-equiv./l.) ($\bar{x}$ ± S.E.)
Brown trout		
F.W.	11	133·3 ± 3·1
½ S.W., 240 hr.	5	131·4 ± 7·8
½ S.W., 240 hr.–S.W., 48 hr.	4	185·2 ± 4·5
½ S.W., 240 hr.–S.W., 240 hr.	8	168·9 ± 6·1
½ S.W., 240 hr.–S.W., 64 days	3	150·0 ± 9·5
Sea trout		
F.W.	2	110, 137
S.W., 24 hr.	2	186, 196
S.W., 5 months	5	138·4 ± 0·8
Brackish, indefinite	7	121·7 ± 1·6

Table 3. *Serum sodium in Scottish brown and sea trout*

State of acclimatization	N	Serum Na (m-equiv./l.) ($\bar{x}$ ± S.E.)
Brown trout		
F.W.	5	149·8 ± 3·1
½ S.W., 240 hr.	5	139·0 ± 10·7
½ S.W., 240 hr.–S.W., 240 hr.	6	179·7 ± 6·9
½ S.W., 240 hr.–S.W., 64 days	3	163·0 ± 6·5
Sea trout		
F.W.	2	141, 168
S.W., 24 hr.	2	203, 206
S.W., 5 months	5	165·8 ± 2·2
Brackish, indefinite	1	144

Table 4. *Serum potassium in Scottish brown and sea trout*

State of acclimatization	N	Serum K (m-equiv./l.) ($\bar{x}$ ± S.E.)
Brown trout		
F.W.	5	2·7 ± 0·5
½ S.W., 240 hr.	5	5·1 ± 0·8
½ S.W., 240 hr.–S.W., 240 hr.	6	1·5 ± 0·2
½ S.W., 240 hr.–S.W., 64 days	3	3·1 ± 0·3
Sea trout		
F.W.	2	4·0, 6·6
S.W., 24 hr.	2	0·5, 1·3
S.W., 5 months	5	3·5 ± 1·5
Brackish, indefinite	1	> 10

DISCUSSION

It seems reasonable to conclude from this that, at least for the populations of sea and brown trout near Aberdeen and for the concentrations measured, there are no significant osmotic or ionic regulatory differences between the two forms. However, the implications of the somewhat anomalous behaviour of serum chloride concentration complicate the situation.

The serum Na:Cl ratios for all groups of brown trout are in the range 1·07–1·13. Excepting the two fish sampled after only 24 hr. in sea water (and therefore probably not in states of internal ionic equilibrium) all groups of sea trout had Na:Cl ratios of 1·18–1·28. This difference in ratios is perhaps indicative of a larger contribution of alkaline reserve to total serum concentration in the sea trout than in the brown trout. Such a difference in alkaline reserve could account for the statistically significant difference found between the mean serum chloride concentrations of brown trout in sea water for 10 days as compared with sea trout in sea water for 5 months. It also might indicate a real difference between sea and brown trout with respect to mechanisms for control of blood acid-base balance.

If correct, this interpretation would perhaps not be in agreement with the results of work on the eel (*Anguilla*) done by Boucher-Firly (1935). Boucher-Firly found consistent decreases of alkaline reserve (to about 40% of mean fresh-water levels) in all life-history stages of the eel following long-term acclimatizations to sea water (more than 12 days). The relative constancy of the Na:Cl ratios in brown trout and sea trout in both fresh and sea water argues against the occurrence of such changes in *Salmo trutta*. However, the actual concentration changes involved would be small (on the order of 10 m-equiv./l.) and may have been masked in the present material by other effects. Direct measurements of alkaline reserve in brown and sea trout are obviously needed.

The only previously published data on osmotic and ionic concentrations in *Salmo trutta* that the writer is aware of are given by Dekhuyzen (1905), Spalding, in Jones (1956), and Phillips & Brockway (1958). Dekhuyzen lists serum Δ for one brown trout in fresh water in the Amsterdam aquarium (−0·57° C.), and a mean for six dead, or nearly dead, sea trout from sea water at Bergen, Norway (−0·78° C.). Spalding lists mean serum Na and K concentrations for brown trout in fresh water during the winter (Na: 144·2 m-equiv./l.; K: 8·9 m-equiv./l.). Phillips & Brockway (1958) present figures for the means and ranges of Cl, Na and K concentrations (also concentrations of several other ions) in the serum of brown trout in fresh water in summer. These values are: Cl: 119 m-equiv./l. (range: 118–124 m-equiv./l.); Na: 156 m-equiv./l. (range: 151–162 m-equiv./l.); K: 5·2 m-equiv./l. (range: 4·3–6·6 m-equiv./l.).

The present data on serum Δ and Na in fresh water agree well with the figures of Dekhuyzen and Spalding, the K data do not. The cause of the discrepancy is unknown. The Δ from dying or dead sea trout is probably unreliable.

The figures given by Phillips & Brockway (1958) are different from both the present data and those of Spalding, probably primarily as a result of having been obtained from summer fish. Brown trout in summer are quite different from brown trout during the rest of the year (Gordon, 1959, for details).

The patterns of variation in serum concentrations indicated here for Scottish brown trout are similar to those found in American brown trout. The latter are discussed in detail elsewhere (Gordon, 1959). However, there are several points which should be made here concerning comparisons between the two groups of fish.

As noted, the overall patterns of variation of the different serum components are very similar in the two groups, though there are small quantitative differences. This may be seen for serum Cl by comparing Table 5 with Table 2. Table 5 presents serum Cl data for brown trout from a hatchery in Massachusetts acclimatized in the ways noted. The experiments were carried out during the same times of year as the experiments with Scottish fish. The only significant difference between the two series is that the American experiments were done at 20° C., the Scottish at 10° C. Additional data on the American fish show that this temperature difference results in the American figures averaging 10 m-equiv./l. higher than they would have been if the experiments had been carried out at 10° C.

Table 5. *Serum chloride in American brown trout*

State of acclimatization	N	Serum Cl (m-equiv./l.) ($\bar{x}$ ± S.E.)
F.W.	14	140·5 ± 1·1
$\frac{1}{2}$ S.W., 240 hr.	12	148·2 ± 2·3
S.W., 21–30 hr.	15	203·6 ± 3·3
$\frac{1}{2}$ S.W., 240 hr.–S.W., 47 hr.	5	170·4 ± 1·6
$\frac{1}{2}$ S.W., 240 hr.–S.W., 240 hr.	7	179·1 ± 11·5

This agreement in overall patterns of regulation indicates that conclusions concerning mechanisms of ionic regulation in the brown trout reached on the basis of the American experiments are very likely valid for the species as a whole. The quantitative differences between the two groups indicate that their many generations of genetic isolation from one another have resulted in small amounts of divergence, but not in any basic changes.

The two groups of data also demonstrate that the brown trout is an almost perfect osmotic and ionic regulator. With long-term acclimatizations over the range of salinities from fresh water to full sea water (again excepting serum Cl after 10 days in sea water) all internal concentrations were either statistically insignificantly different from the fresh-water levels or were within 10 % of the fresh-water levels. Many individual trout were completely homoiosmotic. *Salmo trutta* is the first salmonid known to possess such excellent regulatory abilities (cf. Gordon, 1957).

Finally, there is a small but interesting point of difference between American and Scottish fish with regard to the degree of parallelism shown between changes in serum Δ and changes in serum Cl and Na. In American brown trout under all the conditions studied serum Cl contributed 40–45 %, serum Na 45–49 % of the Δ (assuming unit activities for the ions). Much the same was true for the Scottish fish, but with one clear exception. This was the group of sea trout sampled from brackish water in an estuary. In these fish ion concentrations were significantly below the range to be expected from the Δ measurements (only enough material for one Na determination was available). Cl made up only 35 %, Na only about 41 % of the total concentration.

It might be worthwhile investigating the possibility that, in fish subjected to continually and widely varying salinities, some blood component other than Na and Cl becomes important in regulation of total osmotic pressure. The one serum K value from a fish in brackish water was well above the calibration range of the analyses. Perhaps K is the other component varied (though this would seem unlikely on the basis of known depressant effects of high external K concentrations on nerve and muscle function). This situation may be similar to the differential behaviour of serum Δ and serum Cl noted in shallow-water fishes in Hebron Fjord, Labrador, in winter by Scholander *et al.* (1957).

SUMMARY

1. Adult brown trout (*Salmo trutta* L.) of both sea-run (sea trout) and fresh-water stream (brown trout) forms were captured in the vicinity of Aberdeen and acclimatized to full-strength sea water for periods of up to 5 months.

2. Blood serum samples from these fish were analysed for freezing-point depression, chloride, sodium and potassium concentrations.

3. The patterns of regulation of these concentrations are very nearly the same in both forms. Brown trout and sea trout, at least in eastern Scotland, thus appear to be virtually identical in osmotic and ionic regulatory abilities. However, there is a possibility that there is a difference between the two forms with respect to mechanisms controlling blood acid-base balance.

4. The patterns of regulation shown by Scottish fish are the same as those shown by American hatchery fish treated similarly. The different populations of the species seem not to have diverged significantly from one another in this regard after many generations of more or less complete genetic isolation.

5. The species *Salmo trutta* is strongly homoiosmotic. Internal concentrations are either unchanged or increase by less than 10% above fresh-water levels with long-term acclimatizations to half and full sea water. The brown trout is the first salmonid species known to regulate so well.

These studies were aided by National Science Foundation Predoctoral Fellowships for 1954–57 and a grant from the Higgins Fluid Research Fund, Yale University. This paper is a portion of a dissertation presented to the faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy. Contribution number 927 from the Woods Hole Oceanographic Institution.

The author's thanks for aid and advice are due to the following: Drs E. J. Boell, T. H. Waterman, G. E. Pickford and L. M. Passano, Yale University; Drs C. O'D. Iselin, B. H. Ketchum and A. C. Redfield, Woods Hole Oceanographic Institution; Dr H. Barnes, Marine Laboratory, Millport, Scotland; Mr K. A. Pyefinch, Brown Trout Research Laboratory, Pitlochry, Scotland; Dr I. W. Smith, Marine Laboratory, Aberdeen, Scotland; Dr H. O. Werntz, Harvard University; and Mr H. Robbins, Wareham, Mass.

REFERENCES

ALM, G. (1949). Influence of heredity and environment on various forms of trout. *Rep. Inst. Freshw. Res. Drottning.* **29**, 29–34.

BOUCHER-FIRLY, S. (1935). Recherches biochimiques sur les teleostéens apodes (Anguille, Congre, Murène). *Ann. Inst. oceanogr. Monaco,* **15**, 217–327.

DEKHUYZEN, M. C. (1905). Sur la pression osmotique dans le sang et dans l'urine des poissons. *Arch. néerland. Sci.* **10**, 121–36.

GORDON, M. S. (1957). Observations on osmoregulation in the Arctic Char (*Salvelinus alpinus* L.). *Biol. Bull., Woods Hole,* **112**, 28–33.

GORDON, M. S. (1959). Ionic regulation in the brown trout (*Salmo trutta* L.). *J. Exp. Biol.* **36**, 227–52.

JONES, I. C. (1956). The role of the adrenal cortex in the control of water and salt-electrolyte metabolism in vertebrates. *Mem. Soc. Endocrin.* no. 5, 102–20.

NEAVE, F. (1944). Racial characteristics and migratory habits in *Salmo gairdneri*. *J. Fish. Res. Bd., Can.* **6**, 245–51.

PHILLIPS, A. M., JR. & BROCKWAY, D. R. (1958). The inorganic composition of brown trout blood. *Progr. Fish Cult.* **20**, 58–61.

SCHOLANDER, P. F., VAN DAM, L., KANWISHER, J., HAMMEL, T. & GORDON, M. S. (1957). Supercooling and osmoregulation in Arctic fish. *J. Cell. Comp. Physiol.* **49**, 5–24.

SKROCHOWSKA, S. (1951). The rearing of sea trout, *Salmo trutta* L., in artificial ponds. *Bull. Int. Acad. Polon. Sci. Lett., Classe Sci. Math. Nat.* Ser. B (II), no. 4–6, 179–226.

SKROCHOWSKA, S. (1953). The migrations of sea trout (*Salmo trutta*) and other salmon fishes bred in ponds. *Polsk. Arch. Hydrobiol.* **1**, 89–135 (in Polish, English summary).

SVÄRDSON, G. (1955). Salmon stock fluctuations in the Baltic Sea. *Rep. Inst. Freshw. Res. Drottning.* **36**, 226–62.

TREWAVAS, E. (1953). Sea-trout and brown trout. *Salmon and Trout Mag.* **139**, 199–215.

STUDIES ON THE SHOALING BEHAVIOUR OF FISH

I. RESPONSES OF TWO SPECIES TO CHANGES OF ILLUMINATION AND TO OLFACTORY STIMULI

By D. M. STEVEN

Department of Zoology, University College of the West Indies

(*Received* 23 *August* 1958)

INTRODUCTION

One of the most striking characteristics of many species of fish is the fact that they live in shoals or schools, and since the fisheries of the world depend in large measure upon this habit it is one of great importance to mankind. Yet it is a subject that has been surprisingly neglected by students of animal behaviour, with the result that our understanding of the mechanism of shoaling has lagged behind the accumulation of empirical knowledge obtained by the application of echo-sounding and other new techniques in fishery research. These, in addition to providing us with much new information on the movements of fish shoals in the sea, may suggest problems that are suitable for experimental study.

Considerable advances have also been made in recent years in our knowledge of the sensory capabilities of fish and of the physiology of their nervous systems; and this knowledge too suggests new approaches to the problems of shoaling behaviour.

Most studies on shoaling have been concerned with a single aspect of the subject, such as the sensory basis of the mutual attraction of fish to one another, and there has been as yet no attempt to describe the whole behaviour pattern of a pelagic species in terms of the various external and internal stimuli that are of significance to the fish. As a contribution to this end the following paper describes observations on the responses of shoals of two species of fish found in Caribbean inshore waters to changes of light intensity and to olfactory stimuli associated with food.

MATERIAL AND METHODS

The fish used for the studies here described were the silversides, *Hepsitia stipes* (Müller & Troschel), and the tomtate, *Bathystoma rimator* (Jordan & Swain), which are common in shallow water around Jamaica and the tropical Western Atlantic. Both species live in shoals but have somewhat different habits in other respects. Silversides fulfil the criteria of an 'ideal' shoaling species as defined by Parr (1927); that is to say they are always found in shoals made up of individuals of nearly uniform size; the two sexes are indistinguishable by any external character; if the shoal is made to disperse it, nevertheless, reforms again at once, and the fish feed exclusively on plankton. They show no sign of any territorial behaviour and most of the time swim in midwater. I have never seen individuals of this species pick up food from the bottom. In many respects therefore the behaviour of silversides resembles that of the pelagic Clupeidae.

The tomtate is mainly a plankton feeder but will also pick up food from the bottom and will attack anything that is not too large for it. Shoals in the sea tend to be associated with features in the submarine landscape, such as the piles of wharves and coral formations, and when alarmed the whole shoal retreats into the most sheltered corner of its territory. The sexes are similar in external appearance but the individuals in a shoal vary in size more than do the silversides. It may be considered representative of a demersal type of shoaling species.

The silversides used in these experiments were 5–7 cm. in length, the tomtates 7–11 cm. Immediately after capture the fish were placed in the experimental aquaria and left there undisturbed for a few days. Silversides formed a shoal within a few minutes and appeared to have accustomed themselves to life in the aquarium within 24 hr. Tomtates usually remained in a corner on the bottom for 2 or 3 days and did not form a shoal which swam freely around the aquarium until 5–7 days after capture. They were fed two or three times weekly on small pieces of beef liver or fresh clam meat and occasionally on live plankton. Small shoals of silversides could be kept for several weeks without special feeding, the fish presumably obtaining sufficient food to maintain themselves from the water supply to the aquarium.

The shoals were discarded and replaced by fresh ones after each series of experiments, which usually lasted from 2 to 4 weeks. This was done to minimize conditioning to their food and other possible changes in behaviour resulting from long periods of confinement, and to permit experiments to be repeated with freshly caught fish.

The experimental aquaria and the arrangement of their water supply are shown diagrammatically in Figs. 1 and 2. Two glass-sided aquaria, A and A' 150 × 38 cm. and 38 cm. deep, were mounted side by side and supplied continuously with fresh sea water from a common reservoir, R. A master tap, T_1, controlled the overall rate of flow and the delivery rate to each aquarium could be adjusted by the taps T_2 and T_2'. The system was balanced so that both aquaria received the same amount of water at all times, although the total rate delivered to each aquarium varied between 1·7 and 2·4 l./min. according to the head of water in the supply reservoir. The water was discharged into the aquaria close to one end by a number of small holes bored through 15 mm. internal diameter polythene tubing, the end of which was plugged, and the outlet was by an overflow pipe at the other end. With this system the water drifted slowly through the aquaria from the inlet to the outlet and the mechanical effect of small changes in the rate of supply was negligible. Glass taps and T pieces and polythene tubing were used throughout the system.

For testing the reactions of fish to substances in solution a 1 l. aspirator bottle, B and B', was connected with the delivery tube to each aquarium and mounted just above the water level so as to drain into the aquaria when the control taps, T_3 and T_3', were opened. Narrow bore taps were used here and the system was adjusted to deliver 100 ml. from the bottles into the aquaria in about 60 sec. The rate of delivery

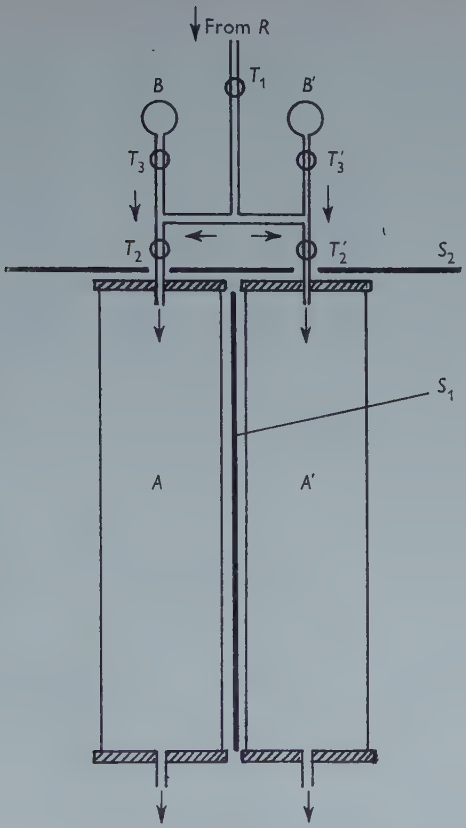


Fig. 1. To show the arrangement of the experimental aquaria, A and A', and their water supply. Water was delivered by gravity from a common reservoir, R. Delivery to each aquarium could be controlled by the taps T_2 and T'_2; the total delivery rate by T_1. B and B' are 1 l. aspirator bottles connected with the supply tubes to the aquaria. Taps T_3 and T'_3 control the delivery rate from these bottles. S_1 is a screen placed between the aquaria so that fish in one tank could not see those in the other, and S_2 another screen serving to conceal the experimenter from the fish. Not to scale.

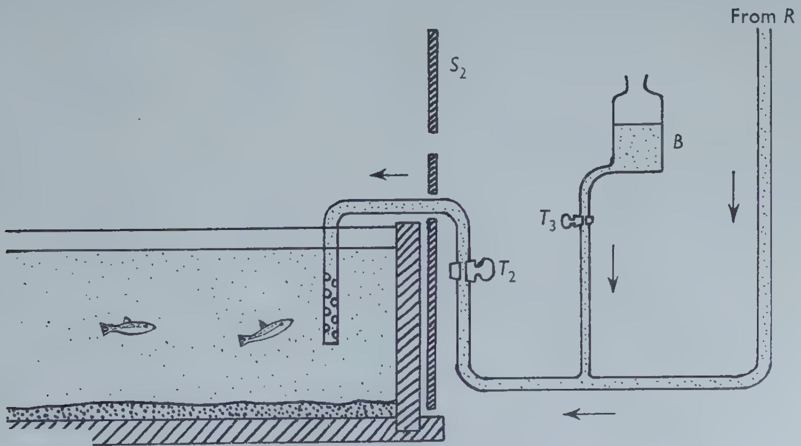


Fig. 2. To show the arrangement of the injection apparatus of one aquarium. Explanation of the lettering is given in the legend of Fig. 1.

from the bottles varied a little with the head of water in the common supply reservoir, but never exceeded an increase of 8% in the total rate of supply to the aquaria. Control tests carried out with sea water in the bottles showed that the behaviour of the fish was not affected by an increase of this amount in the rate of flow. To check that no back flow took place from either bottle into the aquarium on the opposite side of the common supply line the system was tested with solutions of potassium permanganate and indian ink, which showed that all the liquid from each bottle was carried with the supply current into the aquarium with which it was directly connected. The time interval between opening tap T_3 and the appearance of coloured liquid at the inlet to the aquarium was 15–18 sec. and there was good mixing with the sea-water supply during passage down the common part of the tube.

Solutions for testing were made up in sea water from the same source as supplied to the tanks and care was taken to see that it was delivered at the same temperature, which varied from 25 to 27·5° C.

It is not possible with this system to estimate the concentrations of substances in the aquaria, where they were diluted rapidly in a much greater volume of water, but results can be expressed in terms of the total amount of substance delivered and, knowing the rate of supply from the bottles and reservoir, the concentration at the inlets to the aquaria can be estimated.

A removable screen, S_1, was placed between the two aquaria so that the shoals were invisible to each other and each aquarium received illumination only from above and along one glass side. Another screen, S_2, which projected about 50 cm. above and beyond the aquaria, was mounted across the inlet end. The control taps and injection bottles of the supply system were attached to the outside of this screen, which was also provided with a peephole above each aquarium. The experimenter could manipulate the taps and observe the reactions of the fish from behind this screen without being seen by them.

Levels of illumination were measured with an Eel photometer, the photocell of which was mounted pointing upwards just above the surface of the water. During the daytime light from the windows of the laboratory entered through the exposed glass face so that light intensities were more or less uniform throughout the aquaria.

Swimming speeds were measured from 16 mm. ciné films of the fish taken through the open glass side of an aquarium against a 10 cm. square grid marked on the background screen, S_1. Illumination was by 48 in. fluorescent strip lights mounted a few inches above the surface of the water and augmented by two 40 W. Photofloods. Film was shot at 16 frames per second and swimming speeds calculated by counting the number of frames exposed while a fish travelled in a horizontal direction across one or more successive squares. Measurements were made only of fish which appeared to travel parallel with the grid and at right angles to the optical axis of the camera, i.e. in which there was no apparent change of size or of focus while crossing a square. The camera was mounted approximately 150 cm. from the middle of the long side of the aquarium and filming was limited to the four grid squares immediately facing that point, which gave a maximum angle of about 6·5° on either side of the optical axis. Estimates of swimming speeds have not been corrected for the degree of parallax or for the distance between the fish and the background grid, since the errors involved are of minor order.

BEHAVIOUR OF SHOALS BY DAY AND BY NIGHT

Silversides and tomtates showed similar diurnal changes in their patterns of shoaling behaviour. During the day, when the illumination at the water surface ranged from 2 f.c. to more than 50 f.c., the fish kept in tight shoals for most of the time near the bottom of the aquaria. They remained more or less stationary for long periods and swam relatively slowly unless alarmed or otherwise stimulated. Average swimming speeds recorded at illumination of 6–13 f.c. were 7·9 cm./sec. for silversides and 12·8 cm./sec. for tomtates.

Movements of a shoal were usually initiated by a single fish, though not always the same one, and the rest of the shoal followed. If, as frequently happened, the others failed to follow it the leading fish soon stopped, turned through 180° and rejoined the shoal. A single fish rarely travelled a distance greater than one and a half times its own length from the main body of the shoal before stopping or turning back.

When the shoal moved it frequently happened that one or more fish remained behind for a second or two. These then swam faster to catch up with the main body and the greater the distance by which they were separated the faster they swam. Fish turning to rejoin or hurrying to catch up with the shoal swam faster than those departing from it.

Swimming speeds of fish within the main body of the shoal were closely adjusted to each other and in general were slower than those of single fish that had become separated from it. Fish did not keep any definite station within a shoal but continually shifted their positions relative to one another, sometimes lagging a little behind or accelerating and slowing up again.

Deviations from the shoal of single fish in lateral or vertical directions were followed by similar corrective movements. Fish that deviated to one side usually made a complete turn through 360° to rejoin the shoal from the rear.

Some recorded swimming speeds illustrating these remarks are given in Table 1.

The conclusion drawn from these observations is that during the day the shoals are kept in being by a balance between the tendency of single fish to depart and go their own way and the mutual attraction they have towards others of their own kind. As Spooner (1931) found with *Morone labrax* the coherence of a shoal is the result of continuous adjustments of the speed and direction of swimming.

At night the shoals were dispersed; the fish swam singly in all directions and at all levels in the aquaria, with no apparent co-ordination of direction or speed. Groups of two or three fish frequently formed for a second or two but dispersed again. Swimming speeds, though not measurable, were markedly faster than by day and certain actions seen rarely or not at all during the day were common at night.

Silversides frequently rose to the surface to investigate or to seize small floating objects, while tomtates repeatedly picked up pieces of matter from the bottom.

To summarize, the fish were more active and did not swim in shoals at night and their behaviour was clearly directed towards the investigation and sampling of potential food.

Table 1. *Swimming speeds of unactivated shoaling fish in daylight*

	Speed (cm./sec.)	
Silversides		
Fish swimming ahead of shoal	4·8	
Same fish rejoining shoal after turning	13·3	
Fish swimming ahead of shoal	5·0	
Same fish rejoining shoal after turning	10·0	
Fish leading shoal	2·5	
Fish catching up with same shoal	12·1	
Pairs of fish in main body of shoal	Fish 1	Fish 2
	4·7	3·8
	4·8	5·2
	4·8	4·0
	8·0	7·3
Tomtates		
Fish swimming ahead of shoal (mean of 12 measurements)	10·3	
Fish catching up rest of shoal (mean of 7 measurements)	25·1	
	Leading fish	Second fish
Pairs of fish in main body of shoal	16·0	13·3
	6·7	6·4
	16·0	13·3
	5·5	7·6

THE CHANGE FROM DAY TO NIGHT PATTERNS OF BEHAVIOUR

Shoals were observed continuously from the onset of twilight to see if there was a sudden switch from the day to the night pattern of behaviour at a certain level of illumination. An electric pocket torch fitted with a deep red filter was used as an aid to observation when it became too dark to see the movements of the fish. This had no apparent effect upon their behaviour.

The behaviour of both species followed a similar course as the light intensity decreased and may be best illustrated by the following typical series of observations of a shoal of eleven silversides.

Time	Illumination (f.c.)	Observations
16.30	36	Door and windows of laboratory open; bright sunshine outside. Fish in tight shoal near bottom; stationary or swimming slowly
16.35	1·2	Door and window closed and shuttered; laboratory diffusely illuminated. Fish in tight shoal
16.40	1·2	Shoal keeping tight but swimming more rapidly from end to end of aquarium than when window and door open
16.48	30	Window and door opened again. Shoal instantly became tighter, ceased swimming from end to end and became nearly motionless close to bottom
16.55	30	Shoal still tight and nearly stationary
16.57	1·1	Door and window closed again
16.59	1·1	Shoal swimming actively once more

(The door and window were now left closed until nightfall)

Time	Illumination f.c.	Observations
17.40	0·6	Fish swimming actively from end to end of aquarium keeping in tight shoal
18.20	0·2	Behaviour as at 17.40
18.40	0·1	Swimming rate noticeably faster; shoal formation loose
18.45	0·07	Shoal dispersing for a few seconds and then reforming; fish swimming in all directions when shoal dispersed
18.52	0·05	Shoal dispersed most of the time; reforming momentarily in loose groups of two or three
18.58	0·05	Fish seen to rise to the surface for the first time; all fish very active; shoal dispersed most of the time
19.05	0·05	Several fish now rising to the surface frequently
19.10	0·05	Fish generally dispersed, mostly swimming rapidly from end to end of aquarium and often rising to surface; small groups still coming together momentarily

The change in behaviour of tomtate shoals, with the onset of night, followed a similar course and need not be described separately, except to record that they rose to the surface less frequently than silversides but investigated the bottom and sides of the tank very actively, picking up and sampling small objects whether edible or not.

These observations show clearly that the change-over from day to night patterns of activity is a progressive one and there is no sudden switch from one pattern to the other at any level of illumination. The changes in the order in which they become apparent may be summarized as follows:

(1) An increase of swimming speed.

(2) An increase in the distance that individual fish move away from the rest of the shoal before turning to rejoin it. This is what distinguishes a loose from a tight shoal.

(3) Dispersal of the shoal with the fish swimming in all directions; at first only momentary but progressively for longer and longer periods until dispersal is general.

(4) Individual fish rise to the surface (silversides) or explore the bottom of the tank (tomtates).

EXCITATION OF FEEDING BEHAVIOUR BY OLFACTORY STIMULI

The responses of fish to substances in solution were studied by introducing small amounts of test substances dissolved in sea water into one aquarium by the injection apparatus already described, while an equal quantity of sea water was injected into the other aquarium. Tests were replicated, the shoal in each tank being used in turn as a control for the other. The behaviour of the fish was watched for at least 2 min. before and 5 min. after each injection. If a response was obtained, it usually occurred within a minute of starting the injection. An interval of an hour or more was allowed between successive tests and substances from which doubtful responses were obtained were retested on another occasion. During the whole series of experiments lasting 8 months each preparation and substance was tested several times with different shoals of both species.

The rests were carried out during the day or with the aquaria illuminated after dark and only with inactive or slowly swimming shoals.

The response to an exciting olfactory stimulus resembled in some respects the normal night-time behaviour pattern and consisted of: (1) an increase of swimming speed; (2) dispersal of the shoal; (3) swimming to the vicinity of the inlet and returning thereto repeatedly; (4) in some cases rising to the surface, picking up objects from the bottom and attacking any small object seen in midwater.

It was possible with tomtates, but not with silversides, to distinguish clearly between two types of response to an olfactory stimulus, namely, the detection of a substance in the water and the activation of the fish to exploratory feeding behaviour. A substance was said to be perceived when (1) there was an initial increase in swimming speed which was not maintained for more than 2 or 3 min., and (2) the fish swam to the vicinity of the inlet and sometimes sampled the water issuing from it.

Fish were said to show exploratory feeding behaviour when in addition to the above (1) fast swimming was maintained for 5 min. or longer, and (2) they repeatedly investigated, picked up and sometimes swallowed pieces of filter-paper and other objects from the bottom.

Table 2. *Swimming speeds of activated fish*

	Number of measurements	Swimming speed (cm./sec.)		
		Mean	Min.	Max.
Bathystoma rimator				
Unactivated	26	12·8	4·0	26·7
After activation by fresh clam juice				
0–5 min.	49	23·7	16·0	60·0
10–15 min.	20	27·0	8·4	40·0
25–30 min.	9	15·7	4·0	48·0
40–45 min.	14	14·1	3·8	32·0
Feeding	33	36·9	16·0	107·0
Hepsitia stipes				
Unactivated	26	7·9	2·5	20·0
After activation by fresh clam juice				
0–5 min.	18	19·9	8·0	53·3

Measurements of swimming speeds of unactivated and activated fish are given in Table 2. Average speeds of fish activated by an olfactory stimulus were approximately double those of inactive ones, while peak speeds were nearly three times as great. The greatest swimming speeds were recorded when fish were in the act of feeding.

Swimming speeds remained high for some time after olfactory stimulation and then declined slowly to about the initial level, which was usually attained in from 15 to 40 min. (Table 2). Shoals reformed during this period.

RESPONSE TO EXTRACTS OF NATURAL FOOD SUBSTANCES

Shoals of silversides and tomtates responded with intense and sustained exploratory feeding activity to dilute sea-water extracts of a number of natural food substances. Extracts were prepared by chopping with scissors or grinding with sand 1 or 2 g. of tissue and allowing it to stand in a small quantity of sea water for a few minutes with occasional stirring. The suspension was then filtered or centrifuged and the filtrate made up to a convenient volume. Extracts of fresh plankton were prepared by filtering, scraping the plankton from the filter-paper, crushing lightly, mixing with more sea water and refiltering. The effectiveness of preparations was assayed roughly by serial dilutions of the initial extract made up to 100 ml. in sea water. All extracts injected into the aquaria were extremely dilute and without colour or odour detectable by the experimenter.

Table 3. *Relative effectiveness of natural food substances in evoking exploratory feeding behaviour*

Preparation	Effectiveness
Fresh plankton	+++
Arca zebra	
Whole animal	+++
Mantle	++
Muscle	+++
Viscera	++
Gonads	++
Diadema antillarum, body fluid	++
Lytechinus variegatus, body fluid	++
Holothuria sp., body-wall muscle	++
Beef liver	
Whole	++
Serum exudate	++
Beef muscle	++
Pork muscle	++
Lamb kidney	+
Bufo marinus, muscle	+
Cod muscle, frozen	+

+++ = intense and long sustained reactions obtained with extreme dilutions.
++ = intense reactions but not from extreme dilutions.
+ = definite reactions but not long sustained nor from great dilutions.

In a preliminary series of experiments it was found that exploratory feeding activity was evoked by almost any 'meaty' extract. A list of substances tested and an indication of their effectiveness is given in Table 3. The most intense reactions were obtained in response to extracts of freshly caught plankton, pieces of the adductor and foot muscles of the lamellibranch *Arca zebra* and the body fluids of the echinoids *Diadema antillarum* and *Lytechinus variegatus*. Some idea of the sensitivity of the fish to these substances is given by the fact that preparations containing the equivalent of 20 mg. of *Arca* muscle evoked a clear feeding response. Assuming the active substance to constitute 0·1 % of the wet weight of muscle, this would represent a concentration of the order of 1×10^{-9} in the water delivered to the aquarium.

Exploratory feeding activity was also obtained in response to filtered sea water which had contained live plankton for a few minutes. Freshly caught plankton consisting mainly of unidentified copepods was brought to the laboratory without delay. Living animals were decanted into a large filter funnel and filtered through Whatman No. 1 filter-paper with continuous addition of fresh sea water. Portions of the animal-free filtrate were then injected into the aquaria.

There was some indication that the effectiveness of preparations was increased by longer contact with the plankton. The shortest period from the collection of plankton to injection of filtrate into an aquarium was 20 min., and 500 ml. of this preparation were required to evoke a response. When, however, the plankton was in contact with the water for 30–60 min., 100 and 200 ml. portions of filtrate were found to be effective.

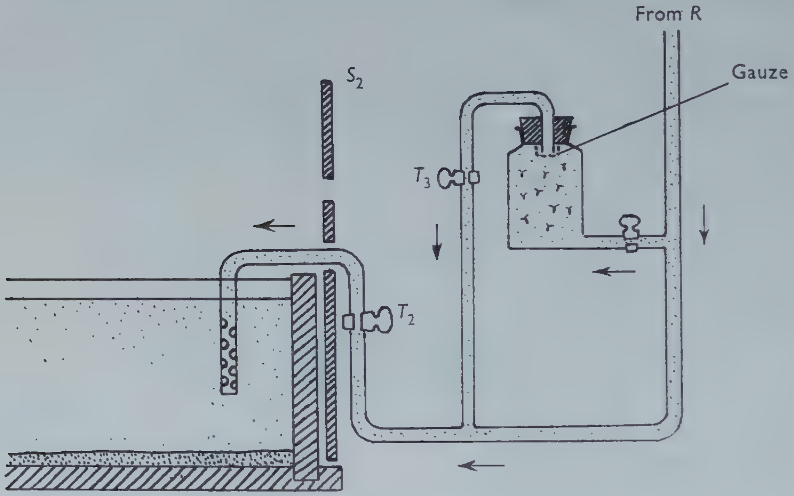


Fig. 3. Modified injection apparatus for introducing an olfactory stimulus from live plankton. Explanation of the lettering is given in the legend of Fig. 1.

To test the possibility that the fish were responding to substances originating from dead plankton organisms killed during the manipulations involved in collection and filtration, the injection apparatus was modified so that a flow of water from the supply reservoir passed through the bottles and thence to the inlet tubes leading to the aquaria as shown in Fig. 3. Live plankton were pipetted into the bottles and were prevented from passing through to the aquaria by a piece of gauze stretched over the outlet tube. After the plankton had been introduced, the bottles were first flushed with several litres of water which were discarded in order to get rid of substances released from dead animals which might have been introduced by the pipetting. Bottles which appeared to contain any dead plankton were not used for experiments. After being flushed out the bottles were connected with the inlet tubes of the aquaria.

Fish responded with exploratory feeding behaviour in most experiments when water from the bottles containing live plankton was first injected into the aquaria, but did not long continue to do so if the flow was maintained. Repeated responses were obtained, however, if the flow through the bottles was interrupted for a few minutes and then restarted.

Although no attempt was made to express the effect of plankton extracts or live plankton filtrates quantitatively, these experiments show that exploratory feeding behaviour may be stimulated by substances released into the sea by these organisms and suggest that they are released continuously.

SIMULTANEOUS PRESENTATION OF OLFACTORY AND VISUAL STIMULI

The reaction of fish to a visual and an olfactory stimulus presented simultaneously was investigated in the following way. A square-sided Perspex column, 45 cm. long and 7·5 cm. side, was placed in each aquarium as shown in Fig. 4. The columns were fitted with two short side-tubes near the base and the top which were connected to the sea-water supply so that water flowed through them and was carried away to a drain without entering the aquaria. A piece of gauze was fitted across the outlets to retain zooplankton. The columns were left in the aquaria containing shoals of six silversides for 2 days before experiments were carried out, so that the fish might become habituated to their presence. At the end of this period they appeared to take no notice of the columns.

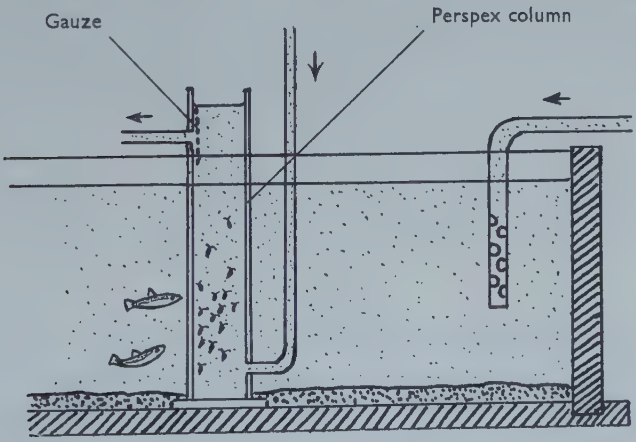


Fig. 4. To show the arrangement with a Perspex column containing live plankton in one aquarium. The sea water circulating through the column does not mix with that in the aquarium.

Live plankton was introduced into both columns and the reactions of the fish observed. Initially the shoals in both aquaria showed great interest in the columns, swam repeatedly around them and returned to them again and again. Frequent attacks on the plankton were observed in which the fish collided with the intervening Perspex or turned aside at the last moment. After a few minutes the attacks became infrequent and the shoals resumed their previous pattern of behaviour, swimming slowly around the aquaria and taking little interest in the plankton, though this was still visible to them in the columns.

An olfactory stimulus consisting of 100 ml. of a filtered extract of plankton was introduced from an injection bottle into one of the aquaria in the usual way about 2 hr. after live plankton was placed in the column, and the attacks made by fish on the column were counted. The fish reacted with intense exploratory feeding activity and during the next 3–5 min. made many attacks on the column. Thereafter, the frequency of attacks declined to approximately the same level as before while the fish resumed their normal unactivated behaviour. A second and a third injection of plankton extract were followed by renewed exploratory feeding behaviour and many attacks on the column. The shoal in the other aquarium reacted in a similar manner to injections of plankton extract. The results of both experiments are shown graphically in Fig. 5.

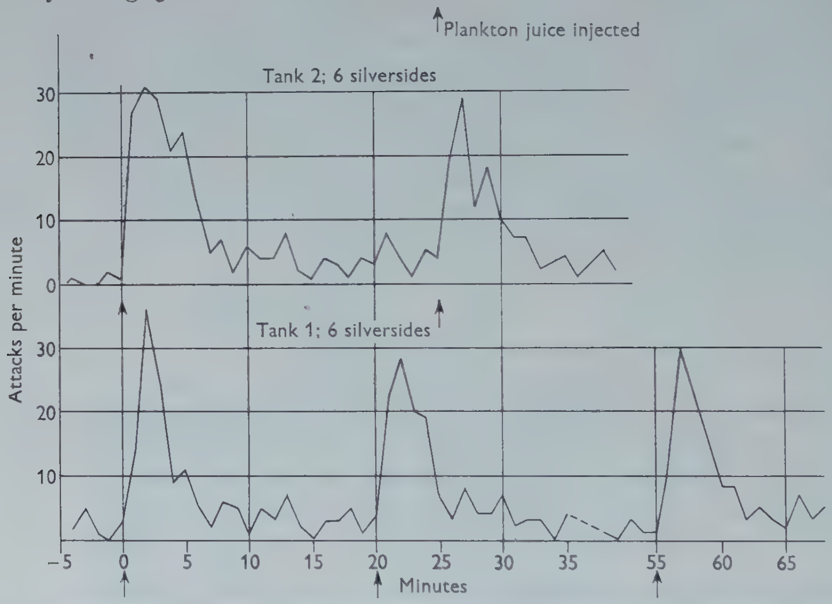


Fig. 5. The frequency of 'attacks' by silversides on plankton in columns. Sea water extracts of fresh plankton were injected at the times marked by the arrows. In these experiments the greatest number of attacks was recorded in the second minute after injection of the plankton extract. This is a consequence of the method of recording. The control tap of the injection bottle was opened 15 sec. before zero time to allow for the passage of extract from the bottle to the inlet of the aquarium. The fish were first seen to react between 18 and 39 sec. after zero time. They were therefore not yet activated for a part of the first minute. In fact the highest frequency of attacks occurred during the first 30–60 sec. after they became active.

Fish activated in the same way by extracts of *Arca* muscle also made numerous attacks on plankton in the Perspex columns.

These experiments show that when excited by an appropriate olfactory stimulus silversides will make visual attacks on objects to which they have previously been negatively conditioned.

FRACTIONATION OF *ARCA* MUSCLE

Extracts of *Arca* muscle were prepared in different ways in an attempt to identify the substance or group of substances that evoked exploratory feeding behaviour.

(1) 2·1 g. of fresh muscle were chopped, ground with sand and boiled for 30 min. in sea water. The mixture was cooled, filtered and the filtrate made up to 250 ml. in sea water. Aliquots of this preparation made up to 100 ml. were injected into the aquaria. Extracts prepared in this way evoked feeding responses in dilutions containing the equivalent of 40 mg. of muscle; they were therefore to all intents and purposes as effective as cold water extracts. Extracts prepared from second and third boilings of the muscle were effective also, but those from the fourth boiling evoked no response.

(2) 4·6 g. of fresh muscle were chopped, ground and dialysed through a cellophane membrane for 15 hr. The dialysate, which had a faintly cloudy appearance, was made up to 250 ml. and serial dilutions of this preparation tested in the usual way. Feeding reactions were obtained from dilutions containing 5 ml. (equivalent to 2 %) of the stock solution of dialysate. The non-dialysable fraction was diluted, filtered, made up to volume and tested in the same way. This too evoked feeding responses in dilutions equivalent to 2 % of the stock solution. Another portion of muscle was dialysed for 48 hr. and the dialysate changed three times during this period. The non-dialysed residue still evoked feeding responses.

(3) 2·8 g. of chopped muscle were extracted successively with a mixture of ether and chloroform followed, by acetone. The extracts were combined and evaporated to a sticky residue which was mixed with sea water. This preparation did not evoke a feeding response. The residue was mixed with sea water, boiled for a few minutes, cooled, filtered, made up to volume and tested in the usual way. Feeding responses were obtained from dilutions equivalent to 10 % of the stock solution.

The conclusion drawn from this series of experiments was that the constituent of *Arca* muscle that stimulates exploratory feeding behaviour is not a protein or a lipoid. It is water soluble, heat stable at 100° C. and has a molecular weight not greater than about 5000.

FEEDING RESPONSES WITH PURE SUBSTANCES

The effect of a number of pure substances in sea-water solution was tested in the hope that one or more might evoke exploratory feeding behaviour similar to that obtained with extracts of muscle or plankton. They were selected on the assumption that a water-soluble constituent of muscle or an excretory product was most likely to yield a positive result. A list of the substances tested and the results obtained are summarized in Table 4. All substances were injected into the aquaria in 100 ml. of sea water and the behaviour of the shoals observed in the usual way. In the case of substances which evoked a response the smallest quantity that was effective is given in the table and for those to which no response was observed the largest amount tested is given.

The results summarized in Table 4 show that a number of substances were detected by the fish, which responded by an increase in swimming speed and congregation around the inlet, but only four substances evoked reactions that approximated to exploratory feeding behaviour as described in a previous section, and in no case was the response so prolonged as with extracts of muscle or plankton. Of these four substances glutamic acid and creatinine evoked feeding behaviour in tomtates only, ammonia in silversides only, while lactic acid was effective with both species.

Table 4. *Response of fish to pure substances*

Substance	Quantity (mg. in 100 ml. sea water) injected into aquarium	Response		
		Substance detected	Exploratory feeding behaviour	Species
Ammonia	10	+	+	*Hepsitia*
Trimethylamine	50	+	—	—
Diphenylamine	350	+	—	—
Urea	100	o	—	—
Histamine	100	?	—	—
Allantoin	Sat. soln.	?	—	—
Guanine hydrochloride	Sat. soln.	o	—	—
Adenine sulphate	Sat. soln.	o	—	—
Xanthine	Sat. soln.	?	—	—
Acetone	1000	o	—	—
Lactic acid	100	+	+	Both species
Creatine	280	+	—	—
Creatinine	12	+	+	*Bathystoma*
Glycogen	100	?	—	—
Sucrose	560	+	—	—
Glucose	780	+	—	—
Glycine	290	?	—	—
L-Tyrosine	250	o	—	—
DL-Methionine	102	o	—	—
L-Arginine	55	o	—	—
L-Proline	90	o	—	—
Glutamine	210	+	—	—
Glutamic acid	40	+	+	*Bathystoma*
Alanine	180	o	—	—
Acetic acid	100	o	—	—
Butyric acid	100	+	—	—

+ = definite reaction. ? = doubtful reaction. o = no reaction.

To test the possibility that some of these substances might represent to the fish only one component of an exciting olfactory stimulus the effect of some arbitrary mixtures was tried. Mixtures containing one or more of the four substances that evoked exploratory feeding in pure solution were usually effective, but not more so than the single substances, and the responses were still of considerably shorter duration than those obtained with extracts of muscle, plankton or other foods.

The conclusion drawn from these experiments is that fish can detect and respond with a change in behaviour to a variety of pure organic substances in trace amounts and a few of these substances may excite a type of exploratory feeding behaviour of short duration.

ACTIVATION OF BLINDED FISH BY OLFACTORY STIMULI

Tomtates were blinded under 1% urethane anaesthesia by making a slit in the side of the orbit, removing the lens and expressing most of the aqueous humour so as to collapse the eyeball. The operation could be performed in less than a minute and the fish made a quick recovery. For several days they were darker in colour than unoperated fish and no longer swam with the shoal but spent most of the time swimming slowly on the bottom or resting in a corner of the aquarium. Silversides did not survive this operation.

Ten fish in all were operated on. Three days after operation four of them showed motor responses to sudden changes of illumination and were destroyed. The remaining six showed no sensitivity to light and were used for subsequent tests.

Single blinded fish were placed in separate aquaria, left for 1 or 2 days and then tested for their response to an extract of *Arca* muscle in the usual way. In every case a characteristic exploratory feeding reaction was observed, which differed from that of normal fish only in the fact that blinded ones remained in contact with or very close to the bottom of the aquarium, swimming in tight circles below the inlet, where they sampled and picked up small objects. When so activated they soon found their way to the vicinity of the inlet from any part of the aquarium.

Their ability to find food was tested by introducing small pieces of *Arca* muscle into the tank, depositing them carefully on the bottom with the least possible disturbance some distance from the fish. Eight such tests were carried out on two blinded fish. In each case the latter became active after a short interval and started to search for the food. The time interval between introducing the muscle and the start of active swimming varied from 7 to 62 sec. Once activated the fish swam along the bottom of the tank and located the general vicinity of the food within a few seconds. They then found it by a series of rapid changes of direction, turning back whenever their course took them further away until eventually they touched the morsel with their snout, when they at once seized and swallowed it.

Blinded tomtates were able also to locate pieces of food suspended in midwater by cotton thread, but usually took longer to do so owing to their tendency to remain in close contact with the bottom.

ALARM REACTION

Silversides gave alarm reactions in response to extracts of fresh skin of one of their own species. A fish was taken from another aquarium, killed and a few milligrams of the skin and scales removed by scraping with a scalpel. The scrapings were shaken with a small quantity of sea water for a few minutes, filtered and made up to 100 ml., which was then injected in the usual way into an aquarium containing a shoal of nine fish.

No striking change in behaviour was seen when the experiment was carried out during the day, when the fish were already in a tight shoal. A sharp increase in the frequency of turning was first noticed 90 sec. after the injection was started. The shoal remained very tight close to the bottom and moved to a position near the outlet end of the aquarium, where it remained more or less stationary for the next 15 min.

A more dramatic result was observed when the experiment was performed at night, the fish being then more active and the shoal dispersed. Two fish first became 'alarmed' 33 sec. after the injection was started and their alarm was communicated to the rest within a few seconds. The shoal reformed and kept in a tight formation close to the bottom for the next 8 min., remaining for most of this time in the middle section of the aquarium. Redispersal began when one or two fish started to swim away from the shoal for a few seconds, though sometimes the rest followed. After about 20 min. the shoal dispersed once more.

Similar experiments with tomtates gave inconclusive results. Sudden changes in the behaviour of the shoal were sometimes observed shortly after injection of the skin extract but there was no unequivocal alarm reaction.

DISCUSSION

It is generally accepted that shoaling fish use their eyes to keep station during the daytime and that blinded fish cannot keep their place in a shoal with unblinded ones, though they may attempt to do so. These facts have tended to divert attention from the part played by other sense organs in the life of pelagic species inhabiting the illuminated waters near the surface of the oceans. It seems to be widely assumed that since these species have for the most part well-developed eyes they search for and capture their food by sight. Consideration of the optical limitations of the fish eye, and the medium in which it operates, suggests that their vision is less satisfactory for finding food than is commonly supposed. The teleost eye is markedly myopic, according to Walls (1942), by as much as 15 diopters, and when in the rest position the lens is accommodated for near vision. It is adapted for seeing objects most clearly at distances of the order of 10 cm. The distance at which any object of a certain size can be seen depends, other things being equal, on the resolving power or visual acuity of the eye. This is best in good illumination and is diminished, though often with a gain in absolute sensitivity, in poor light. There are no reliable data on the visual acuity of fish, but if we assume a value similar to those found for man—it is unlikely to be better—an object 0·09 mm. long should be visible at 30 cm. and one 0·3 mm. at a distance of 1 m. in clear water and good illumination. Bainbridge (1952) found that with practice he could see single *Calanus finmarchicus*, an organism 3 or 4 mm. in length, at a distance of 120–150 cm. under optimum conditions and smaller but opaque species at about 30 cm. It seems unlikely that fish can do much better than this.

Observations made in the course of the experiments described in this paper support the view that fish feeding on plankton use their eyes to detect small organisms in their immediate vicinity only. Silversides were seen frequently to make direct attacks upon small copepods from distances up to about 40 cm., but it could not be shown that they saw their prey at greater distances. On the contrary, they often failed for a considerable period to notice plankton introduced near one end of the aquarium while they were swimming at the far end between 100 and 150 cm. from it.

There is reason, therefore, to doubt whether even the well-developed eyes possessed by most pelagic shoaling fish are of much use for detecting food organisms at distances greater than a metre or so. We have now to consider the evidence in favour of long-distance perception by means of other senses. Tactile and acoustic mechanisms have been relatively little studied, but there is now a considerable body of information on the ability of fish to detect and respond by a change in behaviour to substances in solution. Many fish are known to be extremely sensitive to olfactory and gustatory stimuli, to traces of organic substances and dissolved gases as well as to small differences in the temperature, pH and salinity of water masses. Hasler & Wisby (1951) found that fish may learn to discriminate between and remember odours associated with a particular river, and there is good evidence that certain organic substances may be detected in extremely dilute concentrations. Schutz (1956), for instance, estimated that the 'alarm' substance liberated from the skin of wounded minnows could be detected by other minnows at a dilution of 2×10^{-11}, and many substances have been shown to bring about changes of behaviour at concentrations in the range 1×10^{-8} to 1×10^{-10}. As mentioned above, the substance in *Arca* muscle that excited *Hepsitia* and *Bathystoma* was effective in concentrations of this order at the point of delivery to the aquaria.

There is reason to believe also that olfactory communication plays some part in maintaining the coherence of shoals, since Keenleyside (1955) found that blinded rudd (*Scardinius erythrophthalmus* L.) could detect and preferred water that had contained other rudd to water which had not, but showed no such preference after their olfactory epithelium was destroyed. A recent summary of work in this field has been given by Hasler (1957).

Almost all previous studies of the olfactory sense have been made on freshwater or on non-shoaling marine species, but Tester and his colleagues (1955) found in the flesh and body fluids of tuna and other fish a substance that stimulated feeding behaviour in the predatory pelagic species *Euthynnus affinis* and *Neothunnus macropterus*. They were able to show that the exciting substance was water-soluble and dialysable, at least in part, that it contained phosphorus, the amide link and the benzene ring but not sulphur. They were not able to stimulate feeding behaviour with any of a large number of pure chemicals, though the fish appeared to sense the presence of some of them. Their findings, which were unknown to me at the time, are in general similar to mine with *Hepsitia* and *Bathystoma* and suggest that these pelagic species may hunt their food by following olfactory clues. All we can claim to have demonstrated, however, is that these species can detect very small amounts of certain water-soluble non-protein substances present in a variety of animal tissues and may respond to them with feeding behaviour. These or similar substances are liberated into the water by live zooplankton.

The reaction of *Hepsitia* and *Bathystoma* to an exciting olfactory stimulus has both a kinetic and an orienting component. The former is manifested by an increased rate of swimming as soon as the presence of a substance is detected, the latter by movement towards the source of the stimulus and repeated return thereto. The orienting mechanism was demonstrated most clearly by the manner in which blinded *Bathystoma* located pieces of food introduced into the aquarium some distance from them. The initial swimming response sometimes took the fish towards the food and sometimes further away from it. In the latter case it always turned after travelling a few centimetres and swam in the right general direction. Further turns were made whenever the position of the food was overshot by a few centimetres and the morsel was eventually located by a series of such turns of progressively increasing accuracy.

These experiments have some bearing on the movements of fish shoals in the sea. There is now ample evidence, recently summarized by Hardy (1956) and Lucas (1956), that shoals of plankton-feeding fish aggregate around areas where their principal food is abundant and tend to be absent from areas where food is scarce. The association between North Sea herring (*Clupea harengus*) and *Calanus finmarchicus* has been more intensively studied than any other, but there is evidence of a similar relation between mackerel (*Scomber scomber*) and *Calanus* in the western English Channel and Celtic Sea. It has been shown too that whalebone whales can somehow find areas of high density of the zooplankton on which they feed. It seems reasonable to suppose that shoals can be guided to their food from long distances by olfactory perception and this may well be the most important single factor determining their movements, compared with which differences in temperature, salinity and other characteristics that distinguish different water masses may be of minor significance. We may, in general, expect fish to swim up concentration gradients of substances excreted by their food organisms, and shoals of the same species may find each other by this means. We may also expect them to swim away from alarming or noxious olfactory stimuli and thus avoid unsuitable areas such as those of dense phytoplankton production, though there appears to be still some doubt whether in fact they do so.

In addition to horizontal migrations some pelagic species make regular diurnal vertical movements similar to those of the zooplankton on which they feed. For instance, Balls (1951) and Richardson (1952) have shown by echo-sounding that shoals of herring (*Clupea harengus*) and sprat (*C. sprattus*) rise towards the sea surface at night and descend again to deeper water at daybreak. Richardson believes this behaviour to be a direct response to the change of light intensity, since the vertical movements were observed in non-feeding as well as in feeding fish, but there is no reason to conclude that this behaviour pattern is innate in fish as it seems to be in the case of the invertebrate organisms of the plankton. It seems equally likely that the fish learn to associate the regular diurnal changes in light intensity with vertical movements of their food and, having become so conditioned, continue to respond to the light stimulus even when not feeding. Another possibility is that non-feeding as well as feeding fish may continue to follow the concentration gradient of substances liberated by plankton in their diurnal vertical migrations.

These speculations have now led some way beyond the facts; we clearly need to know more about the nature of all kinds of olfactory stimuli that are of significance to fish, and the way in which associations between different sources of information are formed in the course of their lives. From this type of study we may hope eventually to arrive at an understanding of the behaviour of fish shoals that will enable their behaviour in the sea to be predicted with greater certainty than is at present possible.

SUMMARY

1. *Hepsitia stipes* and *Bathystoma rimator* swim in shoals during the day but disperse at night.

2. The fish become increasingly active as illumination decreases and shoals begin to disperse when it falls below about 0·1 f.c. The change from day to night patterns of behaviour is progressive; there is no sudden switch from one to the other at any single level of illumination.

3. Exploratory feeding behaviour similar to normal nocturnal activity can be induced during the daytime by olfactory stimuli. When so stimulated the average swimming speed is about twice as fast as in unactivated fish.

4. Aqueous extracts of mammalian tissues, of marine animals and water in which live plankton has been kept evoke exploratory feeding behaviour. Fish so stimulated attack live plankton which they can see but not reach and will pick up and sometimes swallow pieces of filter-paper which they had previously ignored.

5. The substance in the adductor muscle of *Arca zebra* that excites exploratory feeding behaviour is dialysable and unaffected by boiling. It is effective in concentrations of the order of 1×10^{-9}.

6. Fish can detect trace amounts of many organic substances, to a few of which they respond in a way similar to exploratory feeding, though of less intensity and shorter duration than is the case with extracts of natural food substances.

7. Blinded *Bathystoma* respond to extracts of *Arca* muscle with a modified type of exploratory feeding behaviour and can locate the position of pieces of food.

8. These experiments are discussed in relation to the behaviour of fish shoals in the sea.

REFERENCES

Bainbridge, R. (1952). Underwater observations on the swimming of marine zooplankton. *J. Mar. Biol. Ass. U.K.* **31**, 107–12.

Balls, R. (1951). Environmental changes in herring behaviour. A theory of light avoidance, as suggested by echo-sounding observations in the North Sea. *J. Cons. Int. Explor. Mer*, **17**, 274–98.

Hardy, A. C. (1956). *The Open Sea.* London.

Hasler, A. D. (1957). Olfactory and gustatory senses of fishes. *The Physiology of Fishes*, **2**, 187–209, ed. M. E. Brown, New York.

Hasler, A. D. & Wisby, W. J. (1951). Discrimination of stream odors by fishes and its relation to parent stream behaviour. *Amer. Nat.* **85**, 223–38.

Keenleyside, M. H. A. (1955). Some aspects of the schooling behaviour of fish. *Behaviour*, **8**, 183–248.

Lucas, C. E. (1956). *Plankton and Fisheries Biology. Sea Fisheries. Their Investigation in the United Kingdom*, pp. 116–38. Ed. M. Graham, London.

Parr, A. E. (1927). A contribution to the theoretical analysis of the schooling behaviour of fishes *Occ. Pap. Bingham Oceanogr. Coll.* **1**, 1–32.

Richardson, I. D. (1952). Some reactions of pelagic fish to light as recorded by echo-sounding. *Fish. Invest. Lond.* Ser. 2, **18**, 1–19.

SCHUTZ, F. (1956). Vergleichende Untersuchungen über die Schreckreaktion bei Fischen und deren Verbreitung. *Z. vergl. Physiol.* **38**, 84–135.

SPOONER, G. M. (1931). Some observations on schooling in fish. *J. Mar. Biol. Ass. U.K.* **17**, 421–48.

TESTER, A. L., VAN WEEL, P. M. & NAUGHTON, J. J. (1955). Reaction of tuna to stimuli, 1952–53. Part 1: Response of tuna to chemical stimuli. *Spec. Sci. Rep. U.S. Fish. Wildl. Serv.* no. 130, 1–62.

WALLS, G. L. (1942). The vertebrate eye and its adaptive radiation. *Cranbrook Inst. Sci. Bull.* no. 19.

SUPERPREGNANCY IN THE MOUSE

1. IMPLANTATION AND FOETAL MORTALITY AFTER INDUCED SUPEROVULATION IN FEMALES OF VARIOUS AGES

By ANNE McLAREN* and DONALD MICHIE†

Royal Veterinary College, University of London

(*Received* 14 *October* 1958)

INTRODUCTION

In 1927 Engle used pituitary implants to induce ovulation and oestrus in adult female mice mated to fertile males. At *post mortem* examination of their uteri 9–10 days later he found large numbers of embryos, ranging from 19 to 29. He did not follow the fate of such embryos into later pregnancy. But his observation raises a question which remains unanswered today: 'Could the reproductive output of mice and other mammals be artificially increased by gonadotrophin treatment?'

Not only adults but also immature female mice respond to pituitary gonadotrophins by the ovulation of large numbers of eggs (superovulation) (Runner, 1950; Runner & Palm, 1953). If treated immature females are paired with fertile males copulation occurs, followed by fertilization and cleavage of the eggs (Runner & Gates, 1954). These eggs can be shown by transfer to uterine foster-mothers to possess normal viability and developmental capacity (McLaren & Michie, 1956; Gates, 1956).

The failure of implantation to occur in immature females following induced ovulation and mating was investigated by Smithberg & Runner (1956). The corpora lutea of the immature animals began to regress 4 or 5 days *post coitum* instead of developing into corpora lutea of pseudo-pregnancy (for terminology see Snell, 1941). Implantation could be induced, and pregnancy maintained to term, by supplying the missing progesterone artificially. The rare animals in which implantation occurred spontaneously were invariably found to have functional corpora lutea.

When pituitary gonadotrophins are administered to adult females without regard to their oestrous phase, a majority respond by superovulation, mating and implantation (Fowler & Edwards, 1957). The proportion of mated females in which implantation occurs is lower than in natural mating. Following implantation, embryonic mortality during pregnancy and parturition is abnormally high, so that the number of young born alive is no greater than normal.

On receiving privately from Drs Fowler and Edwards an account of their work, we made a preliminary study‡ of the ages at which superpregnancy could be induced. In the strains of mice used, superovulation and mating at 30 days of age was regularly followed by pregnancy, although spontaneous sexual activity, and hence the possibility of natural pregnancy, does not appear until some 5–10 days later. We shall refer to this stage as *adolescence*. It is preceded by the *juvenile* stage, studied by Runner & Gates (1954) and by Smithberg & Runner (1956), in which induced ovulation is followed by mating and conception but not by implantation, and is succeeded by the *adult* stage in which oestrus and ovulation occur spontaneously.

---

* Present address: Institute of Animal Genetics, West Mains Road, Edinburgh, 9.

† Present address: Department of Surgical Science, University New Buildings, Teviot Place, Edinburgh 8.

‡ This experiment, described by Michie in the *Journal of the Animal Technicians Association*, 8, no. 1 (1957) suffered from a number of defects and should be regarded as superseded by the present work.

It is clear from the foregoing facts that before we can hope to increase the reproductive yield, or decrease the reproductive age, by artificial means, many questions still require answers. The following are among the questions to which the present work is addressed:

(1) When does adolescence begin?

(2) How do adolescent females compare with adults in their capacity to initiate and maintain superpregnancy?

(3) What is the pattern of embryonic mortality in adolescent and in adult superpregnant females?

(4) Are the large prenatal losses in superpregnancy due to the excessive number of embryos carried or to some other attribute of the experimental treatment?

The experiment to be described also provided information on embryonic growth. The presentation of this material will be deferred to a later paper.

MATERIAL AND METHODS

The mice belonged to a random-bred colony of Theiler's Original strain (TO strain), maintained at the National Institute for Medical Research, Mill Hill, London. All females were virgin. They had been reared on the M.R.C. diet 41, but were fed throughout the experiment on diet 86 of the Rowett Research Institute, supplemented with mixed grain. Ovulation was induced by the intraperitoneal injection of 0·2 i.u. of pregnant mare's serum ('Gestyl', manufactured by Messrs Organon Ltd.) per g. body weight at about 5 p.m. 2 days before pairing with males, followed by the intraperitoneal injection of 0·2 i.u. of human chorionic gonadotrophin ('Pregnyl', manufactured by Messrs Organon Ltd.) per g. body weight at 12 noon on the day of pairing. Females were weighed individually immediately before the first injection, and hormone doses were calculated from these weights.

Treated females were paired singly with males of proved fertility. The occurrence of mating was detected by the presence of a vaginal plug the following morning. The females were thereafter stored in groups of five in larger cages. Untreated control females were also kept in groups of five, with a fertile male in continuous residence. They were examined daily for vaginal plugs.

Seven days after the finding of a plug a laparotomy was performed. The uterine horns were exposed through a ventral incision and the number of swellings in each horn was counted. Care was taken to handle the uterus, and especially the swellings, as little as possible. We have found that this operation neither interferes with the maintenance of pregnancy nor increases embryonic mortality. In normal pregnancies the number of swellings counted at 7½ days *post coitum* agrees well with the number of implants found in the same animal at 18½ days. When the number of swellings is increased by superovulation, the 7½-day count becomes more difficult. Occasionally two embryos which have implanted very close together are contained in a single swelling. In cases of extreme crowding the whole uterine horn becomes thickened and the individual swellings are poorly defined. Where there is early embryonic mortality the 18½-day count is also less reliable. None the less, our 7½-day and 18½-day counts tallied in the great majority of cases (see Table 1).

Table 1. *Comparison of numbers of implants in individual uterine horns, as counted at laparotomy 7½ days* post coitum, *and again at autopsy 18½ days* post coitum

(The data in the upper section are from the experiment described in the present paper; the data in the lower section refer to the preliminary study mentioned above. Uterine horns in which no implants remained at 18½ days have been omitted.)

Ovulation	7½-day exceeds 18½-day count by			7½-day equals 18½-day count	18½-day exceeds 7½-day count by		
	3 or more	2	1		1	2	3 or more
Spontaneous	0	0	0	18	0	0	0
Induced	0	3	14	62	11	2	1
Spontaneous	0	0	0	8	0	0	0
Induced	1	0	2	9	0	1	0

Most of the pregnant females were killed at 18½ days *post coitum*, i.e. shortly before term. Their uterine contents were then classified into live embryos and dead embryos, and the latter were subdivided into early, middle, and late deaths. A few females were found at 18½-day autopsy to show the white blotches on the liver characteristic of Tyzzer's disease. These animals have been excluded from the data.

The classification of embryonic death into *early*, *middle* and *late* was made as follows. Implants which were represented at 18½ days by nothing more than small brown wart-like objects were described as early deaths. These wart-like objects, which we shall term 'moles', are quite distinctive and are familiar to all who have dissected pregnant mice. They have been variously referred to as 'moles' or 'solid moles' (Snell & Picken, 1935; cited by Grüneberg, 1952, p. 144), as 'dead implantation sites' (Grüneberg, 1952, p. 500) and as 'deciduomata' (Bateman, 1958), a term which calls attention to the overwhelming predominance of maternal tissue in these structures. Like Bateman, we have found no evidence of any tendency for the deciduoma to shrink and disappear by resorption after the death of the embryo. In the present work, and also in unpublished material, we have found that if an embryo lives long enough to cause a localized swelling at 7½ days it is always represented by a recognizable relic at 18½ days. We are here referring to normal pregnancies ('spontaneous ovulation' in Table 1). In superpregnancies, other factors, as explained earlier, affect the accuracy of the $7\frac{1}{2}$-day count.

The stage at which early death, as defined above, occurs does not seem to be very accurately or certainly known. Bateman (1958) cites Russell, Russell & Kimball (1954) as believing that it occurs before $10\frac{1}{2}$ days. He himself considers that such embryos have been dead 'long before $10\frac{1}{2}$ days and probably since implantation began' (i.e. since 5 days). Both Russell *et al.* and Bateman were considering early deaths induced by X-irradiation of the father's testes, but they presumably intend their statements to hold for the early deaths occurring in their control material. In the case of early deaths among the offspring of males carrying X-ray-induced translocations Grüneberg (1952, p. 500) says: 'The bulk of the embryonic mortality...happens after implantation, presumably during the formation of the germ layers' (i.e. about 7 days). We shall give the period of early death the relatively non-committal designation of < 10 days.

Any implant which was represented at $18\frac{1}{2}$ days by a well-formed (but typically undersized) placenta without a recognizably formed embryo was classified as a middle death. We assume that these placental relics correspond to deaths occurring after day 9 (otherwise there would not be a well-defined placenta) and before day 13 (otherwise there would be, as we have inferred from our experience with other material, an embryo of grossly recognizable bodily form). However, we emphasize that these assumptions are based on informed guess-work only, and may possibly be in error by as much as one day in either direction.

The third category of embryonic death which we distinguished was based upon the recovery at autopsy of an embryo with recognizable bodily parts. We take such embryos to have died at some time after 12 days.

RESULTS

The main experiment

The vital statistics of the experiment are set out in Table 2, which shows the numbers and fate of the females allotted to the various groups. It presents a number of striking features, which are depicted in Fig. 1.

The effect of age on the occurrence of mating

In the four groups of immature females, the proportion which mated following treatment rose from 11/18 and 10/18 in the two youngest groups to 15/16 and 16/17 in the two oldest. This trend is significant at the 1% probability level as judged by a χ^2 test for regression (Fisher, quoted by Holt, 1948). Evidently in the youngest groups there is a proportion of mice in a pre-juvenile stage in which oestrus, or at least mating behaviour, cannot be evoked by the gonadotrophin treatment.

The mating rate, having attained a peak at 30 days, falls to a lower level among the treated adults. The observed proportion, 30/53, differs from the 30-day value, 16/17, at the 1% level of significance as assessed by Fisher's exact method. The possibility suggests itself that in some of the adults a clash may occur between the treatment and the phase of the spontaneous oestrous cycle in which the female happens to be at the time of the treatment. But in a more recent experiment, in which the oestrous phase at PMS injection was determined in 60 treated adults by the vaginal smear method, the mating rate was found to be independent of oestrous phase.

Table 2. *The vital statistics of the main experiment*

	Aeg (days) at pairing in the various groups					
	Immature superovulated				Adult super-ovulated 50–71	Untreated controls 40–98
	24	26	28	30		
Mean weight (g.) at P.M.S. injection	11·0	11·9	13·3	14·6	20·4	—
No. of treated females	18	18	16	17	53	—
No. with vaginal plugs	11	10	15	16	30	14
No. with 7½-day swellings	2	7	14	16	29	12+1*
No. dying between 7½ and 18½ days	0	0	0	1	0	0
No. with embryos all gone at 18½ days	0	4	1	3	4	2
No. with embryos all dead at 18½ days	1	0	2†	0	1	0
No. parturient at 18½ days	0	0	1	2	1	1
No. with vaginal plugs which had live embryos at 18½ days	1	3	10	10	23	10
No. without vaginal plugs which had live embryos at 18½ days	0	0	0	0	3	—
Total no. with live embryos at 18½ days	1	3	10	10	26	10

* 7½-day laparotomy omitted in error.

† One of these females was seen on day 17½ to have sustained a severe tail injury and has therefore been omitted from the data on foetal mortality.

The effect of age on the occurrence of implantation

We have defined adolescence as the stage in which induced ovulation and mating are followed by implantation, but spontaneous sexual activity does not yet occur. The onset of adolescence can be accurately charted from the data of Table 2. In the 24-day-old group, implantation occurred in only 2 out of 11 mated females, as judged by the presence of 7½-day uterine swellings. The proportion rose rapidly, via 7/10 in the 26-day-old group and 14/15 in the 28-day-old group, to 16/16 in the 30-day-old group. The average age at which adolescence begins in this particular strain can be estimated from these data as about 25 days. It should, however, be emphasized that females of the TO strain mature unusually early. It is clear, for example, from Smithberg & Runner's (1956) work that there are strains in which adolescence does not begin until after 35 days of age.

The number of implants in relation to age

The mean numbers of implants in the various groups, as judged by $7\frac{1}{2}$-day uterine swellings, are given in Table 3. It is clear that there is no difference between adolescent and adult treated females. Both show slightly more than twice the mean number of implants found in untreated control females. The consistent slight excess of the right horn over the left in mean number is in our experience a usual feature of normal mouse pregnancies also.

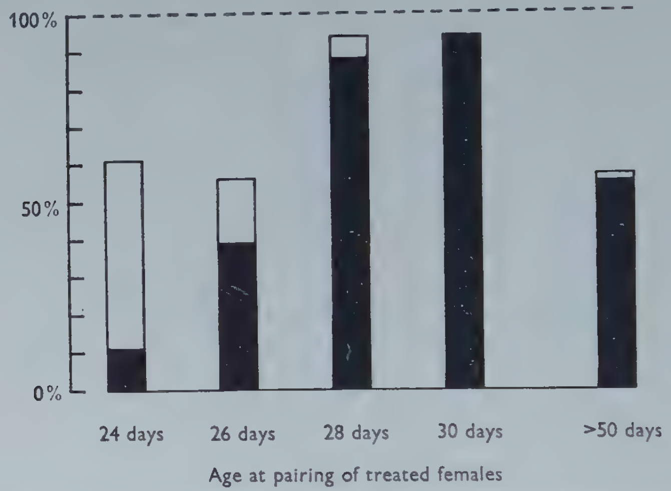


Fig. 1. Mating and pregnancy rates in female mice of different ages treated with gonadotrophins. The total height of each age-block represents the percentage of treated females which showed copulation plugs; the height of the black portion represents the percentage of treated females in which implantation occurred.

Table 3. *The mean numbers of $7\frac{1}{2}$-day swellings in experimental and control pregnancies*

Age at pairing	No. of pregnancies	Mean no. of $7\frac{1}{2}$-day swellings		
		Right horn	Left horn	Total
24 days	2	11·5	10·5	22·0
26 days	7	10·0	7·2	17·2
28 days	14	9·9	8·6	18·6
30 days	16	10·9	9·9	20·7
All adolescents	39	10·4	9·3	19·7
Treated adults	29	10·0	8·9	18·9
Adult controls	12	5·4	3·7	9·2

A very striking difference, however, is seen between adolescent and adult treated females in the amount of scatter about the mean. This is shown graphically in Fig. 2. A possible explanation may again be sought in the existence of a spontaneous oestrous cycle in the adult, but not in the adolescent, females. If the treatment happens to be in phase with the spontaneous cycle of the female, the effects of the treatment may be accentuated; but if it clashes with the spontaneous cycle, its effects may be reduced. In a random assortment of females some will be at a favourable, and others at an unfavourable, phase of oestrus when the treatment is applied, while others will occupy a neutral position. The net result will be an increased variability in the responses to the treatment, as compared with adolescent animals, but no shift in the mean.

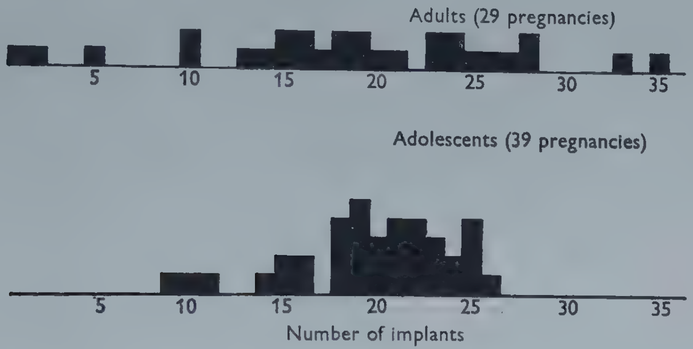


Fig. 2. Distribution of the number of uterine swellings at 7½-day laparotomy in adult and adolescent female mice treated with gonadotrophins.

Table 4. *Analysis of variance of the number of implants (7½-day swellings) in each uterine horn of the treated females*

	Sums of squares	Degrees of freedom	Mean square	Components of variance
Treated adults				
Within females	151·50	29	5·2241	5·2241
Between females	958·72	28	34·2400	14·5080
Total	1110·22	57		$r = +0{\cdot}73$
Treated adolescents				
Within females	246·50	39	6·3205	6·3205
Between females	326·33	38	8·5876	1·1335
Total	572·83	77		$r = +0{\cdot}15$

If this explanation is correct we expect that the whole of the observed difference in variability will be due to greater variation from female to female, and not at all to any difference in the amount of side-to-side variation. We can put the matter to test by performing an analysis of variance, taking individual uterine horns as the units of analysis. The results, shown in Table 4, accord fully with this expectation. The mean square estimating the variance within mice (i.e. between the two uterine horns of the individual mice) is approximately the same in the two groups, the adolescents actually having a slightly higher value. But the 'between mice' mean square for the adults is about 4 times the corresponding value for the adolescents

($P < 0.001$). To bring out the full force of the comparison, the 'between mice' item is also represented in Table 4 by the appropriate component of variance from which effects of side-to-side variation are entirely eliminated. The 'between females' component of variance for the adults is 12·8 times that for the adolescents.

A more familiar, but less informative, way of expressing these results is to say that there is a greater positive correlation between sides in adult than in adolescent implant numbers. The ratio of the 'between mice' to the 'within mice' mean square is in fact equal to $(1+r)/(1-r)$, where r is the intraclass correlation coefficient. The values of r derived in this way are given in the table.

The spontaneously ovulating controls had a similar 'within females' mean square (8·3333) to that of the treated groups, but a very small 'between females' item (2·1664). The corresponding correlation coefficient is −0·59. This presumably reflects the negative correlation between the numbers of eggs shed from the two ovaries in spontaneous ovulations in the mouse (Bowman & Roberts, 1958).

The effect of the age of the female and the number of implants on the maintenance of pregnancy

Turning back to Table 2 we see that a proportion of pregnancies failed, either by the disappearance (presumably by abortion) of the embryos between day 7½ and day 18½ or by the presence at 18½ days of dead embryos only. In Table 5 we have tabulated the frequency of failure in relation to the female's age and to the number of 7½-day uterine swellings. There is a suggestion that the younger females are least able to maintain pregnancy to term. But the chief factor responsible for the loss of the experimental pregnancies seems to be the number of implants. The table shows that in the treated groups the mean number of 7½-day uterine swellings is consistently higher in treated females which subsequently lost their pregnancies than in their more successful contemporaries. The phenomenon is especially marked in the three youngest groups.

Table 5. *Failure of pregnancy in relation to the age of the female and the number of 7½-day uterine swellings*

(Only females which underwent laparotomy at 7½ days are included in this table. Females parturient at 18½ days are considered to have maintained their pregnancies.)

Group		No. of pregnancies	No. failed	Mean no. of 7½-day swellings in	
				Failed pregnancies	Maintained pregnancies
Treated adolescents	24 days	2	1	25·0	19·0
	26 days	7	4	22·5	13·7
	28 days	13	2	24·0	17·9
	30 days	15	3	23·0	20·7
Treated adults		29	5	20·6	18·5
Untreated adult controls		12	2	8·0	9·4

Foetal mortality

The average numbers of live and dead embryos in those females which carried at least one live embryo until autopsy at 18½ days are set out in Table 6 and summarized in Fig. 3. The adolescents are seen to be at least as capable as the treated adults of maintaining large numbers of embryos alive. Indeed, the treated adults inflicted a mortality rate upon their embryos which is about 1½ times that found in the adolescents, and succeeded in bringing, on average, 2·7 fewer live embryos to 18½ days. The additional mortality is entirely accounted for by the large numbers of early deaths (< 10 days), which amount to no less than six per pregnancy.

Table 6. *Numbers of live and dead embryos found at 18½ days* post coitum *in females which had maintained their pregnancies*

(The last column gives the total embryonic mortality when females losing all their embryos (counted as 7½-day uterine swellings) are included. Discrepancies in the 'total' column arise from rounding-off errors.)

Females which maintained their pregnancies to 18½ days *p.c.*		Average no. of embryos at 18½ days						Gross mortality (including loss of entire pregnancy) (%)
			Dead					
Age at pairing	No. of pregnancies	Live	Late	Middle	Early	Total	Mortality (%)	
24 days	2*	15·5	0·5	3·0	3·5	22·5	31·1	55·7
26 days	3	12·0	0·0	0·3	0·7	13·0	7·7	72·1
28 days	10	12·9	0·4	1·8	2·7	17·8	27·5	42·9
30 days	10	12·7	0·9	4·7	2·1	20·4	37·7	53·5
All adolescents	25	12·9	0·6	2·9	2·3	18·6	30·7	53·7
Treated adults	26	10·2	0·7	2·0	6·0	19·0	46·2	55·5
Adult controls	10	8·9	0·0	0·3	0·3	9·5	6·3	19·8

* An additional female, not appearing in Table 2, has been included because of the paucity of pregnancy data in this age group. She received the standard treatment during the period occupied by the experiment, but had no laparotomy.

The gross mortality for the various groups is also entered in the table. This is the percentage of *all* implants which failed, for whatever reason, to be represented by living embryos at 18½ days. Thus not only the partial loss but also the total loss (i.e. all embryos gone or dead at 18½ days) of pregnancy contributes to the estimate. It has approximately the same value for the adult as for the adolescent treated females, since the heavier early mortality in the adults is counterbalanced by the higher proportion of total failures of pregnancy among the adolescents.

The occurrence of very large numbers of implants is, as we have said, a major factor in the loss of entire pregnancies. Is it also largely responsible for the partial losses sustained through the death of individual embryos? In examining this question we can distinguish between embryonic deaths occurring during the early, middle and late periods of post-implantation existence. This distinction turns out to be of great importance. To anticipate our conclusions, the incidence of early death bears no relation to the total number of implants either per female or per uterine horn, whereas death at later stages (after 9 days) is found to be strikingly dependent on intra-uterine crowding.

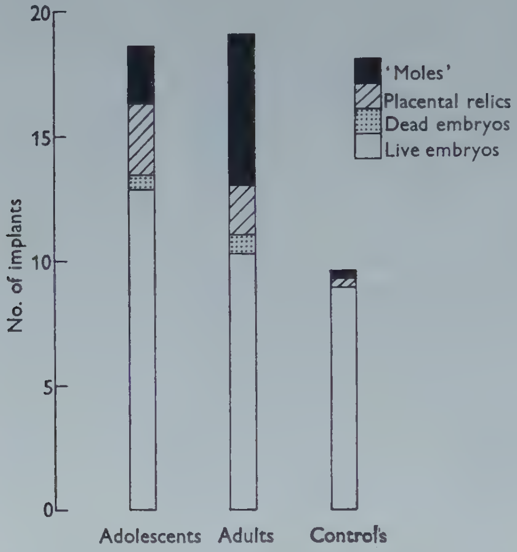


Fig. 3. The average numbers of implants in treated and control female mice which carried at least one live embryo until autopsy at 18½ days. The total height of each block represents the total number of implants per female; this is subdivided into the number of embryos which were alive at 18½ days, and the number which died in early pregnancy ('moles'), middle pregnancy (placental relics) or late pregnancy (dead embryos).

Early deaths

In Table 7 the percentage incidence of early death (< 10 days) is shown after dividing the females into those with small and those with large numbers of implants. It is clear that there is no relation between the early death rate and the number of implants.

The cause of the heavy early embryonic mortality in the treated females, which, surprisingly, affects the adults even more than the adolescents, is obscure. Bateman (1958) says of early embryonic death in the mouse that it 'seems to have few possible causes other than true dominant lethals'. But lethal genes cannot explain the differences shown in Table 7, since the three groups of females and their mates were closely similar in genetic constitution. Bateman also mentions, as a possible rare cause of early death, maternal inadequacy in the development of the deciduomatal reaction at implantation. One might reasonably suppose such inadequacy to occur more frequently after induced ovulation than in normal pregnancy, since disturbances in the luteinization of the ovarian follicles might follow their forced maturation and rupture. In that event, the immediate cause of early deaths would be progesterone insufficiency around the time of implantation. However, in a supplementary experiment where 10 out of 20 superpregnant adults received a subcutaneous implant of 100 mg. of progesterone ('Lutocyclin' implantation tablet, CIBA Ltd.) on day 3 *post coitum*, the incidence of early death showed no significant reduction in the females receiving progesterone.

Table 7. *The incidence of early embryonic death* (< 10 *days*) *in the three groups of pregnant females*

		No. of pregnancies	Early deaths
Treated adolescents	9–19 implants	13	25/206 = 12 %
	20–24 implants	12	32/260 = 12 %
Treated adults	2–19 implants	13	62/176 = 35 %
	20–32 implants	13	94/317 = 30 %
Untreated controls	4–10 implants	6	3/51 = 6 %
	11 implants	4	0/44 = 0 %

There is a third possible cause of early embryonic death, not mentioned by Bateman but suggested by the work of Runner & Palm (1953). This is lack of synchronization between the shedding of the eggs and the arrival of the sperm, resulting in the fertilization of over-ripe eggs which develop for a time and even implant before dying.

Middle deaths

A very different picture emerges when we consider embryonic death during the middle period, namely days 10–12, which is marked by the transference of the embryo's nutritional dependence from the yolk-sac to the true chorioallantoic placenta. It is the persistence of this structure unaccompanied by a recognizably formed embryo which we have taken as our criterion for assigning embryonic deaths to this stage. In Table 8 we have arranged the relevant data according to the number of implants in each uterine horn which survived the first 9 days of life—i.e. the total number of implants less the number of 'moles'. In this way we assess the possible effects of living competitors in the same uterine horn upon an embryo's chances of survival during the middle period.

These effects are seen to be very great. Where the number of implants in a uterine horn which survive to the middle period is 8 or less, the death rate during this period is moderate and does not significantly exceed the control level. But as soon as the number rises above 8, the death rate in both the adolescent and adult treated groups jumps to 20–30 % of all embryos surviving to the beginning of the middle period. The abruptness of the transition is brought out strikingly in Fig. 4. This entire holocaust occurs within a short span of time, probably three days, and must be regarded as a major catastrophe of placentation. It is the more remarkable in that its full force appears to be visited even upon those females which transgress the limit of eight by only one extra live implant, and shows no subsidiary gradation within the '8 or less' and '9 or more' categories. The daily injection of 3·6 mg. of progesterone from day 9 failed to suppress the phenomenon

Table 8. *The incidence of middle embryonic death* (10–12 *days*) *arranged according to the number of implants aged over* 9 *days in the uterine horn*

No. of implants aged > 9 days in horn	10- to 12-day deaths as proportion of all implants aged > 9 days in							
	Treated adolescents			Treated adults			Untreated controls	
1	—	—	6/165 = 4 %	0/5	0 %	10/132 = 8 %	0/2	0 %
2	1/6	17 %		0/6	0 %		0/2	0 %
3	—	—		1/15	7 %		0/9	0 %
4	—	—		2/20	10 %		1/16	6 %
5	0/15	0 %		3/20	15 %		0/20	0 %
6	2/30	7 %		3/30	10 %		1/12	8 %
7	1/42	2 %		1/28	4 %		1/21	5 %
8	2/72	3 %		0/8	0 %		—	—
9	15/63	24 %	66/244 = 27 %	9/45	20 %	43/205 = 21 %	—	—
10	7/30	23 %		11/50	22 %		0/10	0 %
11	24/88	27 %		6/22	27 %		3/92 = 3 %	
12	10/24	42 %		8/48	17 %			
13	10/39	26 %		5/26	19 %			
14	—	—		4/14	29 %			

in a further batch of 14 superpregnant females. It is worth noting that, despite an aberrant control female which had ten implants in one horn and one in the other, eight is usually the maximum number of implants which a single horn is called on to bear in normal pregnancies.

We may inquire whether the whole of the effect is dependent upon the degree of crowding of the individual uterine horn, or whether it is to some extent also affected by the load carried by the opposite horn of the same female. An appropriate

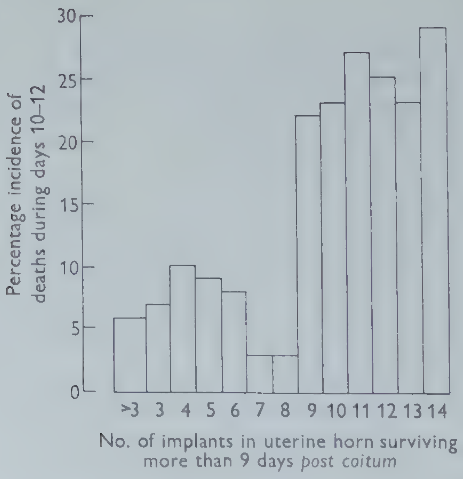


Fig. 4. Incidence of embryonic death during the middle period of pregnancy (10–12 days *post coitum*) in adult and adolescent females treated with gonadotrophins. The number of deaths in each uterine horn is expressed as a percentage of the total number of embryos in the horn which survived till the beginning of the middle period.

arrangement of the data, presented in Table 9, reveals a striking difference between the adolescent and adult treated females in this respect. The latter show no effect of crowding in the opposite horn. But in the adolescent group, doubly crowded females (i.e. with more than eight implants in each horn) have more than three times the middle death rate found in crowded horns when the other horn is *not* crowded. The difference is significant at the 10^{-6} probability level.

Table 9. *The incidence of middle embryonic death* (10–12 *days*) *in treated females, arranged to show the effect of large numbers* (*more than* 8) *of implants aged over* 9 *days in the same uterine horn, and in the opposite uterine horn*

		Opposite horn			
		Adolescents		Adults	
		⩽ 8	> 8	⩽ 8	> 8
Same horn	⩽ 8	2/76 = 3 %	4/89 = 4 %	8/87 = 9 %	2/45 = 4 %
	> 8	19/138 = 14 %	47/106 = 44 %	16/68 = 24 %	27/137 = 20 %

In considering the effect of uterine crowding upon the middle death rate we have ignored possible effects of early deaths. A superficial inspection of our complete data suggested that early deaths might have some protective effect upon the incidence of later death, in that there was a negative correlation between the number of 'moles' in the horn and the proportion of the remaining implants which are found as placental relics at autopsy. Fuller analysis, however, showed this to be no more than an indirect reflexion of the phenomenon which we have just described: horns which had suffered a heavy early death rate tended to have the number of implants surviving beyond 9 days reduced to eight or less, while horns with few or no 'moles' tended to carry larger numbers of implants alive into the middle period and hence to qualify for a heavy middle death rate. After allowance for this, early death was found to have no effect: the occurrence of 'moles' in a uterine horn constitutes in itself neither a good nor a bad prognosis for the survival of the remaining implants.

Late deaths

Compared with early and middle death, the incidence of late death is low, amounting even in the superpregnancies to no more than 9 % of all deaths and 3 % of all implants. None the less, the numbers are sufficient to give a suggestion, as shown in Table 10, that here again the number of embryos carried is a causative factor.

Once again we need to know whether it is the total reproductive load or the local crowding of the individual uterine horn which is primarily responsible. We can answer the question by classifying each of the thirty-three late deaths which occurred in the experimental pregnancies according to whether it occurred in the more crowded or the less crowded of the two uterine horns. For this purpose we have taken as our measure of crowding the number of implants which survived beyond

9 days (i.e. middle deaths + late deaths + live embryos). At the same time we have calculated for each pregnancy the number of late deaths expected to occur in the two horns on the assumption that crowding does not play a causative role. For example, if two late deaths occur in a pregnancy with fifteen implants entering the late period alive, six in the right horn and nine in the left, the expected number of late deaths in the right horn is $2 \times 6/(6+9) = 0{\cdot}8$. The expectations are summed and compared with the corresponding numbers of late deaths which are observed in the more crowded and the less crowded horns respectively. The result is striking and clear-cut, as shown in Table 11. The adolescent and adult females were in close agreement and have been combined. 82% of the late deaths occurred in the more crowded horn, as compared to a chance expectation of 54%. The difference is statistically highly significant. Hence the effect of crowding is to a large extent local, that is, confined to the individual uterine horn.

Table 10. *Mortality among advanced embryos (late deaths) in relation to the number of implants in the mouse which survived to more than 9 days*

No. of implants surviving to more than 9 days	Proportion of advanced embryos dying in	
	Adolescents	Adults
1–14	0/68 = 0%	3/92 = 3%
15–18	5/109 = 5%	6/66 = 9%
19–21	6/121 = 5%	4/48 = 8%
22–24	3/39 = 8%	6/78 = 8%

Table 11. *The tendency for late embryonic death to occur in the more crowded uterine horn*

(Crowding is assessed on the number of implants surviving beyond 9 days. The expectations are calculated from the number of late deaths in each pregnancy and the numbers of implants in the two horns surviving into the late period (> 12 days).)

	No. of late deaths in the two uterine horns of treated adolescent and adult females, based on	
	Chance expectation	Observation
More crowded horn	17·77	27
Less crowded horn	15·23	6
Both horns	33·00	33

$\chi^2_{(1)} = 10{\cdot}39$. $P \ll 0{\cdot}01$.

When middle deaths are not included for the purpose of deciding which of two horns is the more crowded, the effect is still present but is reduced below the level of significance. It is possible that embryos which die during the middle period may effectively compete with their surviving siblings by contributing to intra-horn crowding. This would contrast with embryos dying in the early period, which, as we have seen, do not in any way affect the chances of those which outlive them. Doomed embryos which survive into the period of placentation might perhaps limit the size of the placentae formed by their surviving neighbours. This is in harmony with the parallel finding, reported by Healy, McLaren & Michie (1959), that embryonic growth is retarded by the presence of other embryos in the same horn if, and only if, these survive beyond the early period. We may suppose that limitation of placental size would restrict the rate of uptake of nourishment, and hence diminish embryonic growth, and, in the more extreme cases, viability.

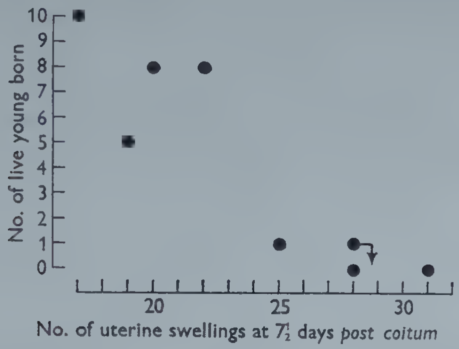


Fig. 5. The number of live young born to adult female mice treated with gonadotrophins, in relation to the total number of implants (uterine swellings at 7½-day laparotomy) in each female. One female which lost its pregnancy through abortion before day 16 has been omitted. The point bearing an arrow relates to a female which appeared ill at full term (20 days) and was therefore killed before parturition had occurred. The uterus contained only one live embryo: this might have failed to survive the hazards of birth.

Parturition in superpregnancy

A further group of adult (6 weeks) TO strain females was allowed to go to term following induced superovulation and mating. 7½-day laparotomy was performed and the number of uterine swellings was recorded. In Fig. 5 the number of live-born young is plotted against the number of 7½-day swellings. A strong inverse relation is seen, showing that birth represents a second major stage of 'density-dependent' loss of young in superpregnancy, the first being the middle period of prenatal life described earlier. When the number of implants is large, the number of young born alive is actually less than in normal pregnancy, a result which calls to mind that of Parkes (1943) who found that the litters produced by adult rabbits following superovulation and mating were abnormally small. It should, however, be pointed out that we used mice which had only just crossed the threshold of sexual maturity, so that their youth may have contributed to the observed effect.

Site of implantation

The relation of embryonic survival to the site of implantation in the uterine horn, independent of intrauterine crowding, has been described and discussed elsewhere (McLaren & Michie, 1959*a*).

DISCUSSION

At the outset of this paper the possibility was mentioned of artificially increasing the reproductive output of mice and other mammals. The question is not entirely academic. The suggestion has been made (Adams, 1954) that the induction of pregnancy in sexually immature animals might be used in livestock farming to diminish the interval between generations. Not only an increase in productivity but also an acceleration of progress in selective breeding could in principle be thereby achieved. Studies have already been made (e.g. Robinson, 1951) of induced superovulation in sheep and other farm animals as a possible means of increasing productivity; these studies have not been extended, so far as we know, to sexually immature females.

In mice we at first thought that the adolescent phase of sexual immaturity, in which induced superovulation and mating is followed by implantation and pregnancy, would prove too early a stage to obtain good yields of live young. It seemed likely that embryonic mortality would prove heavier than in superpregnant adults, and so offset the time gained in using younger females. Our results have shown that this is not the case. Partial loss of pregnancies through the death of individual embryos is in fact somewhat heavier in adult than in adolescent superpregnancies, owing to the greater (unexplained) incidence of early embryonic death (see Fig. 3). The loss of entire pregnancies is commoner in the younger adolescent groups (24 and 26 days old at pairing) than in the adults, but not in the older adolescents (28 and 30 days) (see Table 5). Hence 28–30 days would be the age of choice if we were set the task of extracting the greatest number of live full-term embryos in the shortest possible time out of a given number of TO strain female mice supplied to us, say, at weaning. This choice is re-inforced by considering the proportion of treated females which mate and the proportion of mated females which become pregnant. The mating rate is near to 100% at 28–30 days and falls to below 60% in both the younger and older groups. The pregnancy rate among mated females falls steeply as we reduce the age of pairing below 28 days, but is high in the 28-day, 30-day and adult groups.

A further disadvantage of adult females relative to adolescents is the enormous variation which they show in respect of the number of implants (Table 4). This may reflect heterogeneity either in the number of eggs ovulated, or in the proportion of embryos which implant, or in both. In either case the greater variation in number of implants is undesirable from the practical standpoint.

Even in adolescent females, the death rate at all embryonic stages is very much higher following superovulation than in normal pregnancies. Deficiency of progesterone has been shown experimentally (Smithberg & Runner, 1956; Hall, 1957) to be capable of causing embryonic death at some stages of pregnancy. But in our material progesterone therapy was not effective either against early death or against the catastrophe of placentation which occurs during the middle period when the number of implants in a single uterine horn rises above 8 (Table 8). There is no reason, of course, why progesterone should not alleviate the ill-effects of intrauterine crowding when these are due to sheer compression of the embryo, since the hormone's effect in relaxing the wall of the gravid uterus is well known. For this reason a progesterone supplement might avert *late* deaths in superpregnancy. These resemble middle deaths in being mainly dependent upon the degree of crowding of the single uterine horn. But the existence of a sharply demarcated threshold at eight implants per horn, above which the middle death rate increases abruptly, suggests that the limiting factor may be the amount of uterine surface available for the placentation of a linear series of implants, rather than the overall distension of the uterine horn. In the extreme case the interference between neighbouring sites of placentation is such that placental fusion occurs. The occurrence of this phenomenon in artificially crowded uteri has been made the subject of a separate paper (McLaren & Michie, 1959*c*).

An additional cause of embryonic death during the middle period which remains to be discussed is the double crowding effect displayed by superpregnant adolescents but not by superpregnant adults. Uterine horns in which more than eight implants enter the middle period alive suffer, as we have seen, an increased incidence of deaths in the middle period. But in the treated adolescents this incidence was further greatly increased when the opposite horn also contained more than eight implants surviving into the middle period.

A systemic factor must be sought with effects upon young mice only. An obvious candidate is the nutritional stress involved in supporting the growth of large numbers of embryos while the mother is herself still in the growing period. In support of this idea is the fact that the pregnant adolescent females were invariably found at 18½-day autopsy to have exhausted their reserves of intra-abdominal fat. This was never observed in adult females. On the other hand it is difficult to see why nutritional stress should make itself felt so early in pregnancy. It is here, perhaps, that we may ascribe a role to progesterone insufficiency, arising from a partial failure of adolescent females to form luteal tissue. Such failure is total in pre-adolescent females after induced ovulation and mating (Smithberg & Runner, 1956).

Returning to our imagined task of obtaining the greatest number of live 18½-day embryos from a given number of weanling female mice in the shortest time, we can say that the induced superovulation and mating of 30-day-old adolescents will give yields of about thirteen live embryos per treated female within 3 weeks of weaning the females, as compared with about nine in normal pregnancies within 5–6 weeks. It is worth noting that, despite the effects which we have described of intra-horn crowding upon embryonic mortality in the middle and late periods of pregnancy, we have found no signs of a ceiling to the number of embryos which a female mouse can maintain alive to term. This is brought out in Fig. 6, where we have plotted from our data the average number of live embryos against the average number of implants. The slight tendency shown by the adolescents for the curve to flatten out at the upper end of the range is not statistically significant. In an attempt to find a ceiling using another method, namely the transfer of fertilized eggs, we obtained a similar result (McLaren & Michie, 1959*b*). Robinson (1951), in a study of superovulation in sheep, found that, with increasing numbers of implants, the number of surviving embryos at autopsy reached a ceiling at a level somewhat under four per pregnancy. This is nearly four times the normal litter size of sheep, and so does not necessarily conflict with our findings in mice.

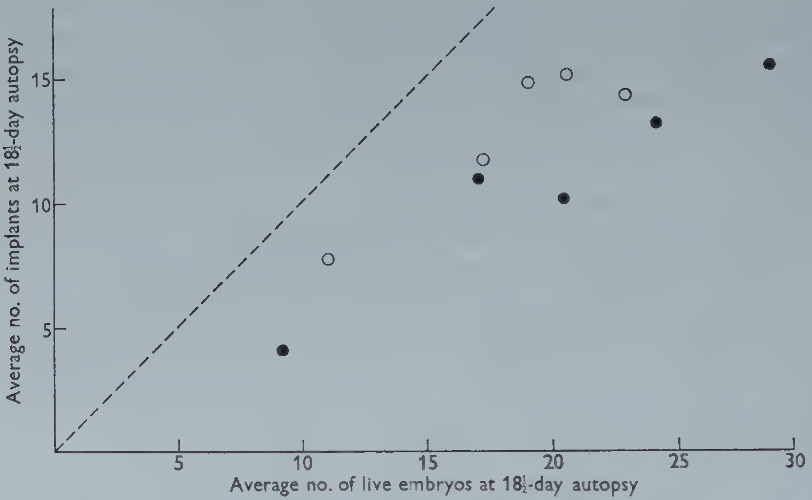


Fig. 6. The number of embryos surviving to 18½-day autopsy plotted against the total number implanting. Each point represents an average derived from four to six pregnancies. Open circles denote adolescent females, solid circles adult females. The broken line sets the limit corresponding to 100 % survival. Females with no live embryos at autopsy have been omitted.

We have as yet said nothing of the greatest hazard of all, namely birth. Our evidence suggests that the greater the number of implants the *fewer* the number of young born alive. The cause of the heavy parturitional losses is unknown.

But even were these losses overcome the survival chances of new-born mice in the large experimental litters might be poor, since such mice are extremely small, see Healy *et al.* (1959). In the pig, increased litter sizes have in the past not been wholly desirable, since the small size and heavy mortality of sucklings in the largest litters more than cancels out the initial gain in numbers. But methods of bottle-feeding piglings from birth have recently been developed, so that it might one day become possible to bring a litter of, say, 20 or more live-born piglings from an induced superpregnancy to marketable age without undue losses.

SUMMARY

1. Superovulation was induced by gonadotrophin treatment in adult and immature female mice. The uteri of treated females and of control, naturally ovulating females were examined at 7½ days *post coitum* by means of a laparotomy operation, and again at autopsy 18½ days *post coitum*.

2. The mating rate of the two older groups of treated immature females was significantly higher than that of either the two younger groups or the treated adults.

The proportion of mated females which became pregnant was low in the youngest group, but increased rapidly with age up to the control adult level.

3. The mean number of implants per uterine horn in treated females in which implantation occurred was about twice that found in untreated control females, and did not vary with age. On the other hand, the *variation* in the number of implants per uterine horn was significantly greater among adult treated than among immature treated females.

4. Total failure of pregnancy through death or disappearance of the embryos was most common among younger females with very large numbers of implants. Partial litter loss through death of individual embryos was greater in adult treated than in immature treated females. Combining partial and total litter loss, the gross mortality rate of embryos was independent of the age of the mother.

5. Death of individual embryos before day 10 was more common in treated than in untreated females, and more common in adult treated than in immature treated females. The incidence bore no relation to the total number of implants either per female or per uterine horn, did not affect the survival of the remaining implants, and was not reduced by progesterone treatment.

6. The death rate of individual embryos during the middle period of pregnancy did not significantly exceed the control level until the number in the horn rose above eight, at which point it increased abruptly in all treated groups. In the immature, but not in the mature, treated females the presence of more than eight implants in *both* horns was associated with still higher mortality.

7. Death of individual embryos during the later part of pregnancy occurred relatively rarely and was associated with crowding of the uterine horn.

8. In adult treated females which were allowed to go to term, the number of young born alive was inversely related to the number of embryos which had implanted.

9. Our results are discussed with reference to the possibility of artificially increasing the reproductive output of mice and other mammals.

We wish to express our thanks to the Agricultural Research Council for financial support, and to Lord Rothschild, F.R.S., for his personal interest in the work.

We are also indebted to CIBA Laboratories Ltd. for supplies of progesterone and for technical advice.

REFERENCES

Adams, C. E. (1954). The experimental shortening of the generation interval. *Proc. Brit. Soc. Anim. Product.* pp. 97–108.

Bateman, A. J. (1958). Mutagenic sensitivity of maturing germ cells in the male mouse. *Heredity*, **12**, 213–32.

Bowman, J. C. & Roberts, R. C. (1958). Embryonic mortality in relation to ovulation rate in the house mouse. *J. Exp. Biol.* **35**, 138–43.

Engle, E. T. (1927). Pregnancy following superovulation in the mouse. *Proc. Soc. Exp. Biol., N.Y.*, **25**, 84–5.

Fowler, R. E. & Edwards, R. G. (1957). Induction of superovulation and pregnancy in mature mice by gonadotrophins. *J. Endocrin.* **15**, 374–84.

Gates, A. (1956). Viability and developmental capacity of eggs from immature mice treated with gonadotrophins. *Nature, Lond.*, **177**, 754–5.

Grüneberg, H. (1952). *The Genetics of the Mouse*, 2nd ed. Hague: Nijhoff.

Hall, K. (1957). The effect of relaxin extracts, progesterone and oestradiol on maintenance of pregnancy, parturition and rearing of young after ovariectomy in mice. *J. Endocrin.* **15**, 108–17.

Healy, M. J. R., McLaren, A. & Michie, D. (1959). Superpregnancy in the mouse. 3. Foetal growth. (In preparation.)

Holt, S. B. (1948). The effect of maternal age on the manifestation of a polydactyl gene in mice. *Ann. Eugen., Lond.*, **14**, 144–57.

McLaren, A. & Michie, D. (1956). Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. I. Factors affecting the implantation and survival of native and transferred eggs. *J. Exp. Biol.* **33**, 394–416.

McLaren, A. & Michie, D. (1959*a*). The spacing of implantations in the mouse uterus. *Mem. Soc. Endocrinol.* No. 6, 65–74.

McLaren, A. & Michie, D. (1959*b*). Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. 2. The effect of transferring large numbers of eggs. *J. Exp. Biol.* **36**, 40–50.

McLaren, A. & Michie, D. (1959*c*). Experimental studies on placental fusion in mice. (In preparation.)

Parkes, A. S. (1943). Induction of superovulation and superfecundation in rabbits. *J. Endocrin.* **3**, 268–79.

Robinson, T. J. (1951). The control of fertility in sheep. Part II. The augmentation of fertility by gonadotrophin treatment of the ewe in the normal breeding season. *J. Agric. Sci.* **41**, 6–63.

Runner, M. N. (1950). Induced ovulations in immature mice as a source of material for studies on mammalian eggs. *Anat. Rec.* **106**, 313–14 (Abstr.).

Runner, M. N. & Gates, A. (1954). Conception in prepuberal mice following artificially induced ovulation and mating. *Nature, Lond.*, **174**, 222.

Runner, M. N. & Palm, J. (1953). Transplantation and survival of unfertilized ova of the mouse in relation to postovulatory age. *J. Exp. Zool.* **124**, 303–16.

Russell, W. L., Russell, L. B. & Kimball, A. W. (1954). The relative effectiveness of neutrons from a nuclear detonation and from a cyclotron in inducing dominant lethals in the mouse. *Amer. Nat.* **88**, 269–286.

Smithberg, M. & Runner, M. N. (1956). The induction and maintenance of pregnancy in prepuberal mice. *J. Exp. Zool.* **133**, 441–57.

Snell, G. D. (1941). 'Reproduction', Chap. 2 in *Biology of the Laboratory Mouse.* New York. Dover Publications.

Snell, G. D. & Picken, D. I. (1935). Abnormal development in the mouse caused by chromosome unbalance. *J. Genet.* **31**, 213–235.

SUPERPREGNANCY IN THE MOUSE

2. WEIGHT GAIN DURING PREGNANCY

By ANNE McLAREN* and DONALD MICHIE†

Royal Veterinary College, University of London

(*Received 17 January 1959*)

(With Plate 4)

INTRODUCTION

In a human pregnancy the average gain in weight is of the order of 20% of the initial body weight (Chesley, 1944). Fekete (1954) weighed female mice daily from the 3rd to the 19th day of pregnancy, and found that the total weight gained during this period varied according to the number of mice in the litter, from an average of 5·5 g. for litters of one mouse up to an average of 19·5 g. for litters of ten mice. The initial body weight of her females varied from 18 to 29 g. The smaller mice would tend to produce the smaller litters (Falconer, 1955), so that the percentage weight gain throughout pregnancy probably ranged from about 30% for small litters up to about 70% for large litters.

Superovulation can be induced both in immature (Runner, 1950) and in adult (Engle, 1927; Fowler & Edwards, 1957) female mice by treatment with pituitary gonadotrophic hormones. Treated females come into oestrus and mate, and if they are adult or at the late stage of immaturity ('adolescence', see McLaren & Michie, 1959), pregnancies ensue in which the average number of embryos which implant in the uterus is more than doubled. Subsequent embryonic mortality is abnormally high, but the average number of embryos surviving to a late stage of pregnancy is still much above normal. In preliminary studies of superovulation and superpregnancy in mice of different ages, the weight gain of the pregnant females was investigated, with results which we present below.

MATERIAL AND METHODS

The mice belonged to the random-bred TO strain (Theiler's Original) maintained at the National Institute for Medical Research, Mill Hill, London. They were fed on diet no. 86 of the Rowett Research Institute.

Nulliparous females of various ages were weighed and given a priming injection of pregnant mare's serum (PMS), followed 43 hr. later (at 12 noon on the day of pairing) by an injection of human chorionic gonadotrophin (CGT). Weights were recorded to the nearest gramme at the time of the PMS injection, and the dose of each hormone was 0·2 i.u. per gramme of body weight. Ovulation is expected to

---

* Present address: Institute of Animal Genetics, West Mains Road, Edinburgh 9.

† Present address: Department of Surgical Science, Teviot Place, Edinburgh 8.

occur 13 hr. after the second injection. The females were paired with males of their own strain, and the occurrence of mating was detected by the presence of a vaginal plug on the morning of the next day (day 0 in our numeration). Control matings of spontaneously ovulating females were also detected by inspection for vaginal plugs.

For purposes unconnected with the present study, the majority of the females were subjected on day 7 to a laparotomy operation in which the uterine horns were exposed and a count made of the number of swellings in each horn. The operation was followed by a transient loss in weight of about 1 g. which was rapidly compensated. No continuing effects were detectable on the weight gain of the pregnant females (see Text-figs. 2 and 3).

From day 7 to day 18 inclusive all females were weighed daily to the nearest 0·1 g. On day 18 all females were killed and their uterine contents were examined. In the case of dead embryos found *in utero*, the approximate stage at which death had occurred was estimated by the criteria used in the previous paper of this series.

Females which died, became ill, lost their pregnancies or were already parturient by day 18 have been omitted.

RESULTS

In order to provide a base-line from which the changes specifically associated with pregnancy can be assessed, the mean weights at various stages of treated females which did *not* become pregnant are given in Table 1. In all age-groups increase of weight occurred during the 10 days elapsing between the injection of PMS and day 7. Thereafter the body weights remained fairly constant irrespective of age, probably as a result of the disturbance and handling of the mice during the daily weighing routine which was instituted on day 7. We do not know whether this effect was due to a suspension of growth or to depletion of fat reserves. In either case it means that we can probably assume that the weight gains of young *pregnant* females between days 7 and 18, to be analysed later, were not complicated by a contribution from the female's own intrinsic growth.

Table 1. *The mean weights of treated TO strain females which did not become pregnant*

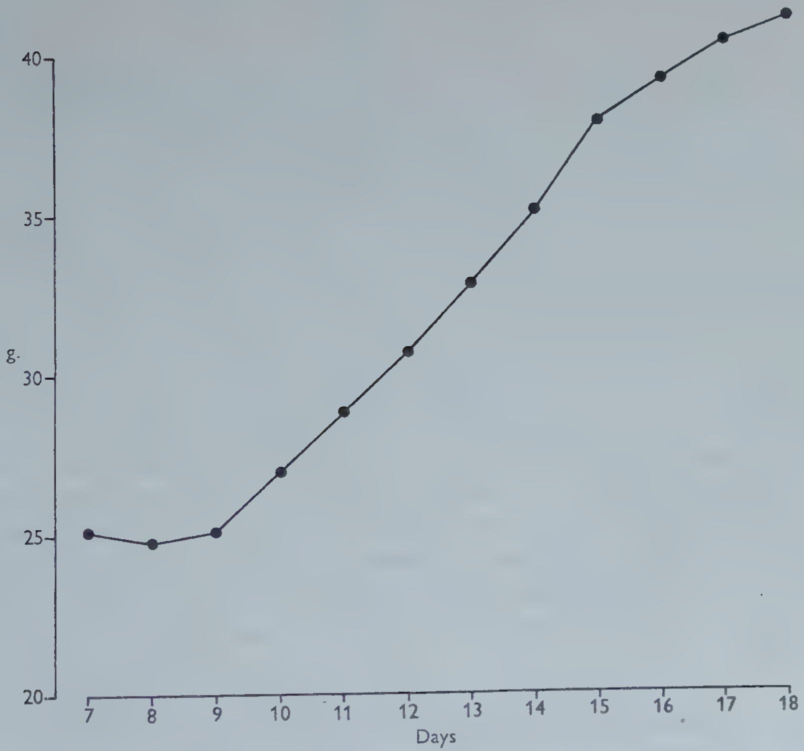
Age (days) at CGT	No. of females	Mean weight (g.) at PMS	Mean 7-day weight (g.)	Mean 18-day weight (g.)
30	2	12·5	16·7	17·1
35	10	15·9	18·6	18·5
40	4	16·0	17·8	18·6
45	5	17·2	19·4	19·1
50	2	19·5	23·3	23·8

The additional weight gain occasioned by pregnancy during the first 7 days can be assessed by comparing the 7-day weights of Table 1 with those of Table 2, in which are listed the mean weights at various stages of the treated and control females which became pregnant, together with the mean number of implants for each group. In the youngest age-group the pregnant females are 20% heavier on day 7 than their non-pregnant contemporaries. The difference is less marked in the older groups, and not correlated with the number of implants.

The changes occurring after day 7 of pregnancy are illustrated in Text-fig. 1, which shows the characteristic form of the curve of weight gain. This curve is from a TO female weighing 19 g. at the time of PMS injection and 50 days old at CGT injection. She was subjected to laparotomy on day 7, found to contain 23 uterine swellings, and killed on day 18, at which time the uterus contained 18 live embryos, 2 which had died between about 10 and 12 days, and 3 which had died earlier. The curve may be divided into three phases: an initial phase, a linear phase and a final phase.

Table 2. *The mean weights of the TO strain females which became pregnant*

Age (days) at CGT	No. of females	Mean weight (g.) at PMS	Mean 7-day weight (g.)	Mean 18-day weight (g.)	Mean no. of implants
30	3	12·7	20·1	26·4	17·4
35	2	16·5	20·5	30·7	15·5
40	1	18·0	21·9	32·2	7·0
45	1	18·0	19·1	28·9	24·0
50	3	19·7	24·5	40·7	23·0
Spontaneous ovulation 63–85	8	—	23·6	36·5	9·4



Text-fig. 1. The growth curve of a superpregnant female mouse from day 7 to day 18 of pregnancy.

In the curve drawn in Text-fig. 1, the initial phase lasts from 7 to 9 days, but in some animals it extends as far as the 12th day. Little if any increase in weight occurs during this period, and there may even be a slight loss of weight during the first day or two. This is probably associated with the change in routine due to the introduction of daily weighing, and is especially marked in those females which underwent laparotomy.

The next phase of pregnancy, during which weight gain is approximately linear, ranges from 3 to 9 days in duration. In the typical case illustrated in Fig. 1 it lasts for 6 days, during which period the mean weight gained per day is 2·15 g. In order to eliminate the element of judgement involved in deciding when this phase begins and ends for each mouse, we have taken the period of 6 days, from day 10 to day 16, as representative of the linear phase for the purposes of our later calculations.

Table 3. *Association between terminal weight loss and late embryonic death in 18 pregnancies*

Late wt. loss	Late death		
	Yes	No	Total
Yes	4	0	4
No	1	13	14
Total	5	13	18

($P < 0{\cdot}002$.)

During the final phase, from 15 to 18 days in Fig. 1, the rate of gain slows down. In some animals this does not occur, and the linear increase in weight is maintained up to day 18. There was an actual loss of weight between days 16 and 18 in four out of ten pregnancies in the treated groups, but not in any of the control pregnancies. As can be seen from Table 3, this terminal weight loss is a reflexion of late embryonic mortality, which is itself associated with uterine crowding (McLaren & Michie, 1959) and therefore more common in superpregnant females.

The effect of the number of embryos carried, and of the initial weight of the female

Maternal weight gain during pregnancy may be expected to depend most closely neither on the *total* number of implants (since those dying early will contribute little) nor on the number of embryos still alive at 18 days, but on some intermediate figure. In considering the effect on weight gain of the number of embryos we have therefore omitted those which were estimated at autopsy to have died before day 13. Henceforward we shall use the term 'embryo' to denote 'embryo surviving to day 13'.

In Table 4 is listed for each female separately the weight gained per embryo during the 7- to 18-day period, together with the 7-day weight of the female, and the number of embryos carried. Inspection of the table shows that the weight gain per embryo decreases with increasing numbers of embryos. There is also an apparent positive correlation with the female's weight on day 7 (this weight to a large extent reflects her age but does not reflect the number of implants). We have used the method of partial regression to disentangle the effects of these two factors. We find that, for a given 7-day weight, the weight gain per embryo during the 7- to 18-day period is decreased by 0·147 g. ± 0·011 for each additional embryo carried, while for a given number of embryos it is increased by 0·155 g. ± 0·025 for each additional gramme in the 7-day weight of the female. Pregnancies following induced ovulation were homogeneous with the adult control pregnancies, and were combined to yield the above estimates.

Table 4. *Weight gain per embryo between days 7 and 18, in relation to 7-day weight of female, and to number of embryos surviving to 13 days*

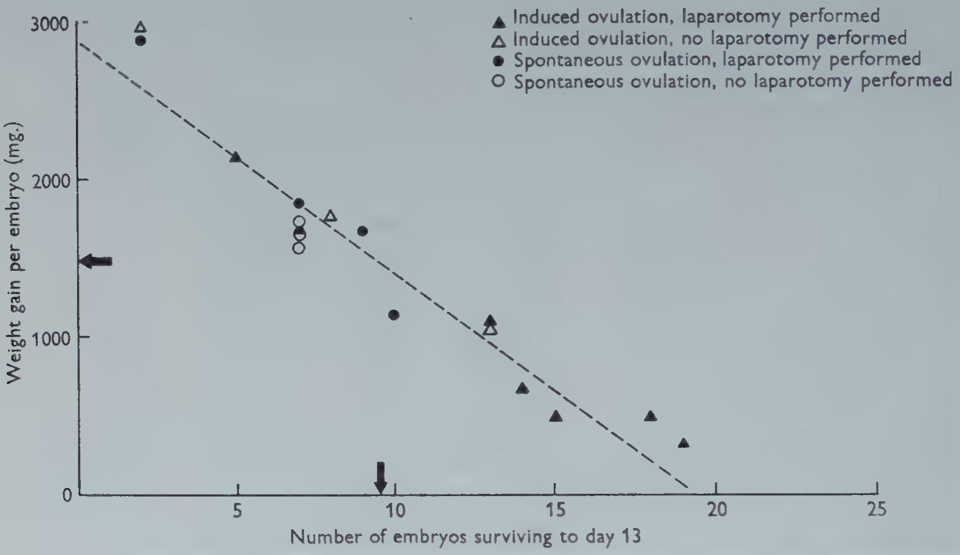
Ovulation	Age (days) at CGT	7-day weight (g.)	Total 7- to 18-day gain (g.)	7- to 18-day gain per embryo (g.)	No. of embryos surviving to 13 days
Induced	30	18·2	7·2	1·03	7
	30	21·0	6·2	0·44	14
	30	21·1	5·5	2·75	2
	35	20·6	10·1	0·78	13
	35	20·5	10·1	0·78	13
	40	21·9	10·3	2·06	5
	45	19·1	9·8	1·22	8
	50	25·1	16·2	0·90	18
	50	24·4	11·8	0·62	19
	50	24·0	10·8	0·72	15
Spontaneous	63	23·3	16·1	1·79	9
	64	24·5	6·4	3·20	2
	69	24·6	14·3	2·04	7
	70	22·7	13·1	1·87	7
	74	22·6	14·4	1·80	8
	80	20·8	9·6	1·37	7
	81	23·7	12·2	1·74	7
	85	26·2	17·1	1·71	10

We can now use the above coefficients of partial regression to eliminate the effects of one variable from the graphical presentation of the effects of the other variable. Thus Text-fig. 2 shows the weight gain per embryo between days 7 and 18, after adjusting to a maternal weight of 22·46 g., the mean 7-day weight of the females. It can be seen that within the range investigated the relation is approximately linear, and varies from about 3 g. per embryo for 2 embryos to 0·3 g. per embryo for 19 embryos.

In the same way we can adjust the data to the mean value of 9·5 embryos, and plot the weight gain per embryo during the 7- to 18-day period against the female's 7-day weight. This has been done in Text-fig. 3. Here again the relation approximates to a straight line, and amounts to a very substantial effect: the weight gain per embryo increases from 0·8 g. up to 2·0 g. as the initial weight of the female varies from 18 to 26 g.

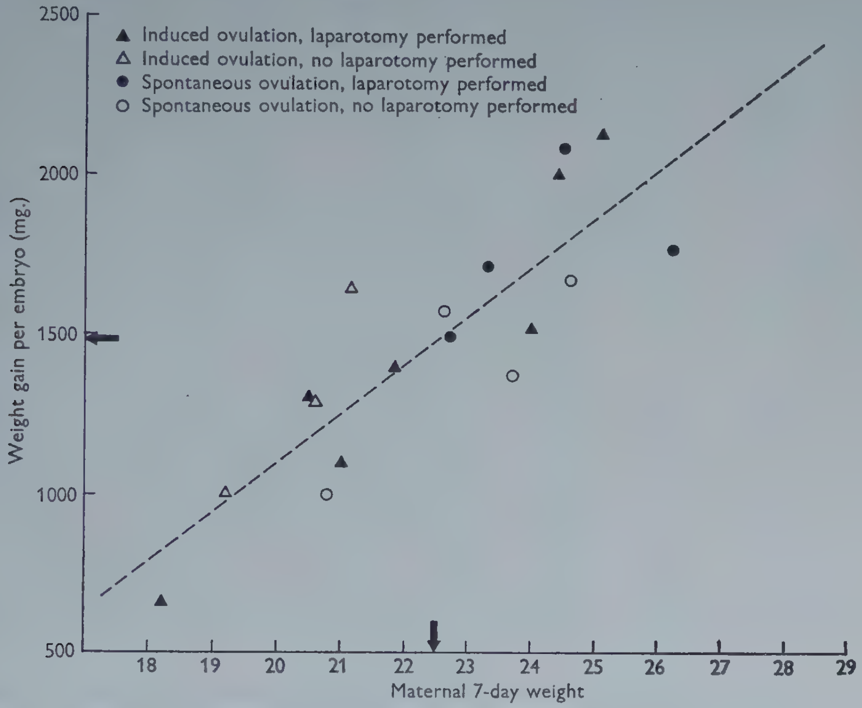
In Text-figs. 2 and 3 the females which underwent laparotomy have been distinguished, and it can be seen that the operation has no consistent effect upon weight gain during pregnancy. Good agreement is also shown between the spontaneous and induced pregnancies.

In order to test the generality of the linear relation between number of embryos and total weight gained per embryo by the female, we analysed the data from a further series of ten TO pregnancies. All of these were young adult females in which ovulation had been induced by the standard gonadotrophin treatment. The mean number of embryos surviving to 13 days was high, and they therefore give useful data on the upper part of the range. Unfortunately the females were weighed on day 10 and not on day 7, but weight gain between days 7 and 10 is small compared with that which occurs later in pregnancy. The partial regression coefficients of 10- to 18-day weight gain per embryo on number of embryos and on 10-day maternal weight were −0·066 g. ± 0·024 and +0·057 g. ± 0·068 respectively. Both regressions were smaller in absolute value than for the main body of data, as might be expected, since 10- to 18-day gains will be smaller than 7- to 18-day gains. The regression on maternal weight is not significantly different from zero, but is in the same direction as before. Text-fig. 4 shows the weight gain per embryo in relation to the number of embryos, after adjusting to the mean 10-day maternal weight (26·8 g.). It will be seen that the linearity of the relation is decisively confirmed.



Text-fig. 2. The relation between the number of embryos surviving to day 13, and the maternal weight gain per embryo between day 7 and day 18, after adjusting to a standard maternal 7-day weight. The broken line gives the calculated slope of the regression. Arrows indicate mean values.

The daily gain per embryo during the 10- to 16-day period of linear weight gain is also dependent upon the number of embryos carried and the 7-day weight of the female. Partial regression analysis on the data from the main experiment gives coefficients of −0·014 g. ± 0·002 and +0·020 g. ± 0·004 for the two variables respectively. This means that for a maternal 7-day weight of 22·5 g., the daily weight gain per embryo during the linear phase varies from about 0·3 g. for 2 embryos down to 0·05 g. for 19 embryos; and increases about three times as the maternal 7-day weight varies from 18 to 26 g.

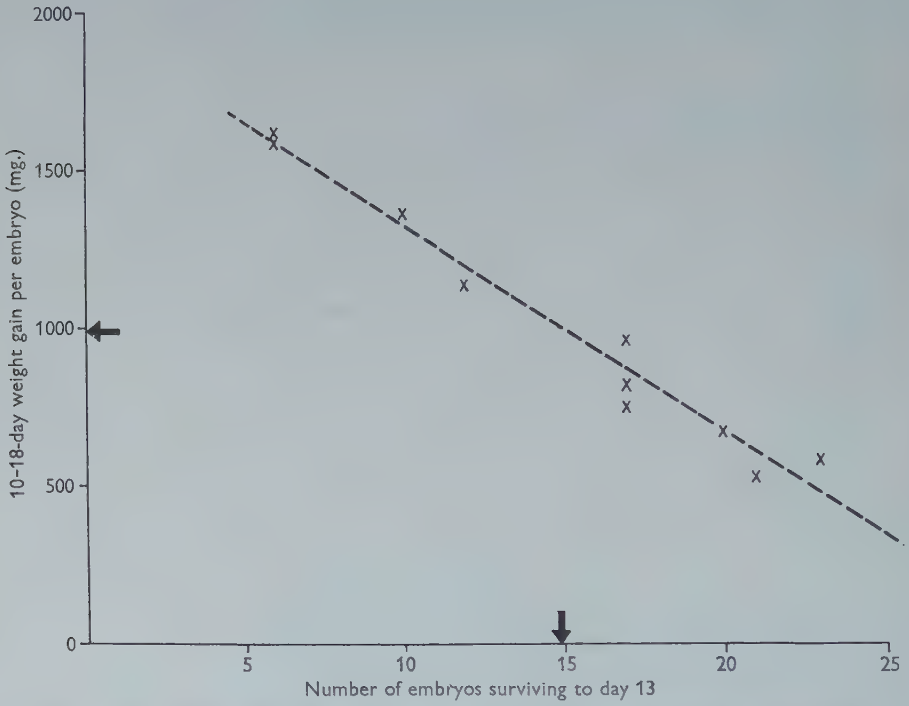


Text-fig. 3. The relation between the maternal 7-day weight, and the maternal weight gain per embryo between day 7 and day 18, after adjusting to a standard number of embryos surviving to day 13. The broken line gives the calculated slope of the regression. Arrows indicate mean values.

The embryonic contribution to maternal weight gain

It is of interest to know what proportion of the total weight gain of pregnant and superpregnant mice is contributed by the live embryos themselves, what proportion by the dead embryos, the uterus and the associated structures (membranes, placentae, amniotic and uterine fluid) and what proportion by extra-uterine weight increase. The main experiment described in the present paper can throw no light on this question, as the embryos were not weighed in isolation from the mother. But in 9 out of the further series, mentioned above, of pregnancies induced in adult TO females, the gravid uterus was weighed at autopsy on day 18 before removal of the embryos, and the live embryos were also weighed. Similar observations were also made on 6 spontaneous and 5 induced pregnancies of adult TO females from the experiment reported in McLaren & Michie (1959).

The relevant data from these pregnancies are given in Table 5. In spontaneous pregnancies the gravid uterus minus the live embryos makes a small and constant contribution, varying from 2·1 to 2·9 g. The much larger and more variable contribution under this heading for the induced pregnancies comes partly from the additional placentae and amniotic fluid associated with the larger number of live embryos and partly from the presence in the uterus of recently dead embryos and the remnants of embryos which have died at earlier stages. It should be added that the weight of the non-gravid uterus is very small in comparison with these values (of the order of 0·1–0·2 g.).



Text-fig. 4. Data from an additional series of TO pregnancies, on the relation between number of embryos surviving to day 13, and maternal weight gain per embryo between days 10 and 18, after adjusting to a standard maternal 10-day weight. The broken line gives the calculated slope of the regression. Arrows indicate mean values.

The importance of extra-uterine weight increase in the spontaneous pregnancies cannot be assessed from these figures, as the untreated mice were not weighed before they became pregnant. In the case of the treated females, Text-fig. 5 shows, for different numbers of embryos, the total weight gain between PMS injection and day 18, divided into weight of live embryos, weight of gravid uterus minus live embryos, and extra-uterine weight gain.

As the number of embryos surviving to 13 days increases from 6 up to 24, the total weight gain increases only slightly, from about 17·5 g. to about 20 g. But the relative contributions of the three components change markedly. The uterine weight gain and the total weight of live embryos both show some increase. The

Table 5. *Data on the embryonic, uterine, and extra-uterine contributions to maternal weight gain in* 6 *spontaneous and* 14 *induced pregnancies of adult TO female mice*

(Females marked with an asterisk belonged to the second series reported in the present paper; the others are from the experiment described in McLaren & Michie (1959*a*).)

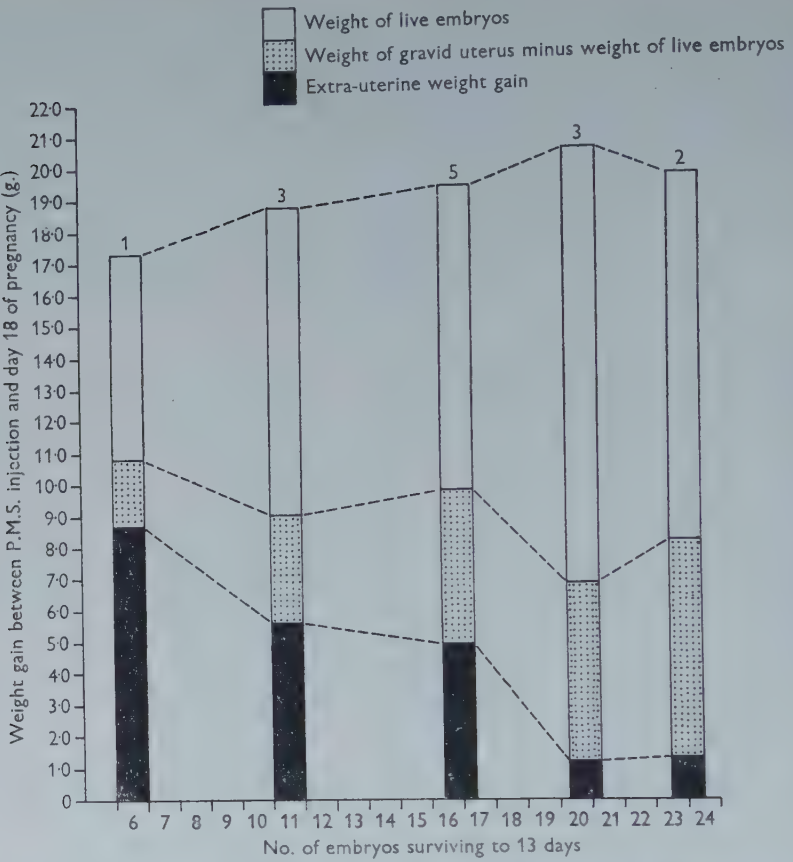
Ovulation	Wt. of ♀ at PMS (g.)	Wt. of ♀ at day 18 (g.)	Wt. of ♀ minus gravid uterus (g.)	Wt. of gravid uterus minus live embryos (g.)	Wt. of live embryos (g.)	No. of embryos		
						Dying before day 13	Dying between days 13 and 18	Alive on day 18
Induced*	19	34·7	20·5	6·10	8·09	6	3	14
Induced*	18	36·3	22·2	4·53	9·58	7	2	15
Induced*	20	37·3	28·7	2·06	6·57	6	0	6
Induced	19	37·3	24·8	4·17	8·31	10	0	11
Induced	21	37·4	20·2	5·36	11·92	6	0	20
Induced*	19	37·5	24·7	2·56	10·20	0	0	10
Induced*	18	38·2	23·0	4·32	10·92	3	0	17
Induced	19	39·5	25·8	5·14	8·57	17	1	14
Induced	18	39·6	25·2	4·60	9·83	8	1	15
Induced*	21	40·4	26·4	3·47	10·57	6	0	12
Induced*	22	40·8	23·1	5·90	11·76	6	2	21
Induced*	22	42·0	23·7	6·65	11·69	7	3	18
Induced	22	42·5	23·6	7·69	11·20	3	5	19
Induced*	23	47·7	25·8	4·91	16·99	2	0	20
Spontaneous	—	28·8	18·7	2·64	7·50	1	0	9
	—	30·8	22·3	2·10	6·38	1	0	7
	—	30·9	20·3	2·42	8·19	1	0	10
	—	33·4	21·4	2·63	9·33	0	0	11
	—	34·9	25·0	2·74	7·18	1	0	8
	—	41·2	28·1	2·87	10·29	0	0	10

failure of the latter to change more strikingly is due to two circumstances: one is the falling proportion of embryos surviving to day 13 which are still alive on day 18 (see McLaren & Michie, 1959); the other is the continuous decline in the average weight of the individual live embryos (Healy, McLaren & Michie, 1959). Over the range 6–24 embryos the contribution of extra-uterine weight gain decreases from about one-half of the total gain to little more than one-twentieth.

DISCUSSION

Fekete's (1954) study was on inbred mice which had been used as recipients in egg transfer experiments and which had ovulated and mated spontaneously. The modal litter size was 3, with a mean of 3·9 and a maximum of 10. Within this range the average weight gain of Fekete's females (day 3 to day 19) was a little larger than ours (day 7 to day 18) for the same litter size.

Fekete's data on weight gain for different numbers of embryos are illustrated in Text-fig. 6. Extrapolating upwards from these data, one might conclude that if the number of mice in the litter were increased above 10, the weight gain per embryo of the mother would not continue to decline, but would approach asymptotically a value of about 1·7–1·8 g. per embryo, while the total weight gain of the mother

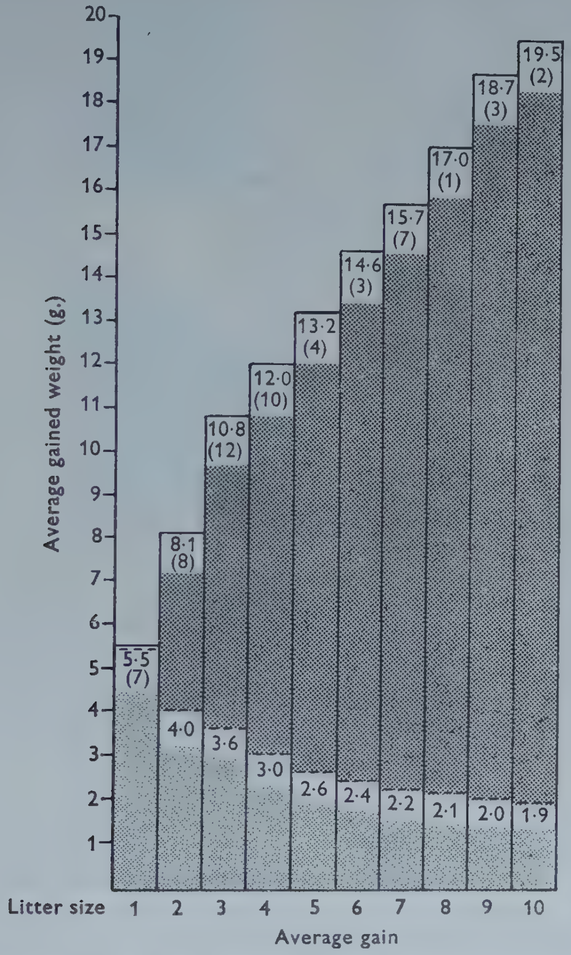


Text-fig. 5. The contributions of weight of live embryos, gravid uterus minus live embryos, and extra-uterine weight gain to the total maternal weight gain between PMS injection and day 18, for different numbers of embryos surviving to day 13. Adult females only. Number above each column denotes number of females in group.

would continue to rise. In this case a superpregnant female carrying 20 embryos would be expected to increase her weight by about 35 g. during the course of pregnancy.

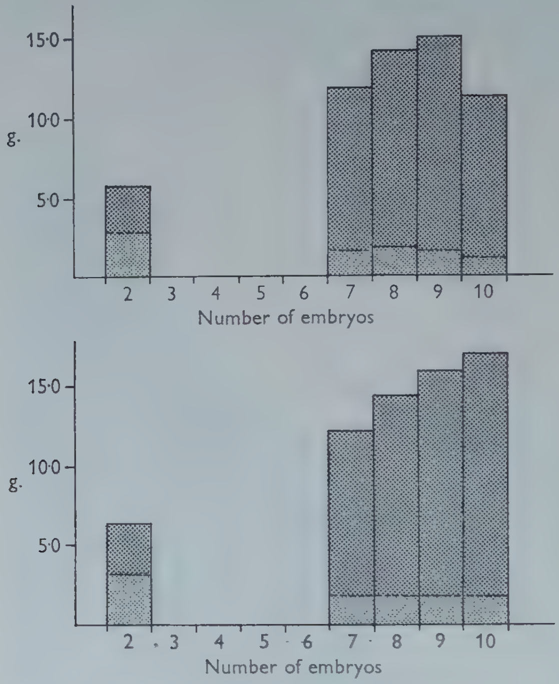
This expectation has not been borne out by the present work. In the last section we saw that total maternal weight gain between injection of PMS and day 18 reached a ceiling of about 20 g.; while it was earlier shown that the weight gain per embryo continued to decrease as the number in the litter increased, and in addition showed a relation with the initial weight of the mother.

The apparent discrepancy between our results and those of Fekete can perhaps be explained if we remember that in Fekete's diagram (Text-fig. 6) no adjustment for the initial weight of the mother has been made. If the data from our spontaneous pregnancies, which cover the same litter-size range as Fekete's, are plotted in the same fashion without adjustment, a diagram closely resembling that of Fekete is obtained, as shown in the lower part of Text-fig. 7. In the upper part of the graph the data have been corrected for the initial weight of the mother by converting our values to weight gained per embryo for a maternal 7-day weight of 22·5 g. (the mean initial weight both of Fekete's and of our females). In Text-fig. 8 the data from the treated females, which are in good agreement after the adjustment for initial maternal weight, have been added. The picture is now quite different. The weight gain per embryo declines persistently as the number of embryos increases; while the total weight gain rises to a maximum and then falls again. Thus for a female of a given 7-day weight, the expected weight gain is *less* if she is carrying 18 embryos than if she is carrying only 9 embryos.



Text-fig. 6. From Fekete (1954). The columns represent the average weight gain (in grammes) during pregnancy of C57BL/6 mice. The lightly stippled areas show the gain per embryo in each litter size group. The numbers in parentheses show how many pregnant females were weighed in each group.

A similar situation obtains in human multiple pregnancies. Stander & Pastore (1940) report that the average weight gain in normal twin pregnancies is less than in single pregnancies.

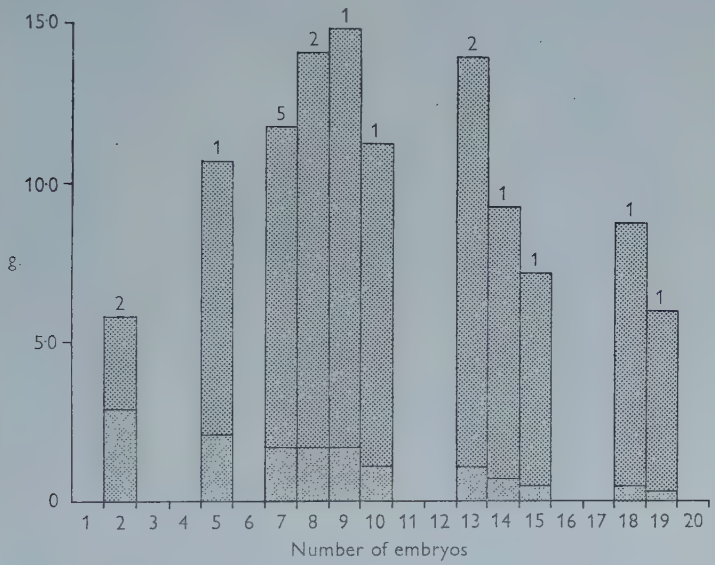


Text-fig. 7. The effect of correcting for the initial weight of the mother. The columns represent the average weight gain of spontaneously ovulating TO females between days 7 and 18 of pregnancy. The lightly stippled areas show the gains per embryo surviving to day 13. In the lower figure, as in Fig. 6, the weight gains have not been adjusted for initial maternal weight. In the upper figure, the calculated partial regression coefficient has been used to adjust the weight gains to a standard maternal 7-day weight.

Although the apparent contradiction with Fekete's results is now resolved, Text-fig. 8 poses a further paradox. For 19 embryos, the maternal weight gain per embryo is seen to be 0·3 g. The regression line fitted to the same data in Text-fig. 2 gives a theoretical value of 0·1 g. per embryo for 19 embryos. Even supposing that the linear relation on which the regression line is based no longer holds in this part of the range, the empirical value of 0·3 g. is still considerably less than the expected 18-day weight of a *single embryo* in a litter of 19 (Healy, McLaren & Michie, 1959), without allowing for the weight increase of the uterus and the extra-uterine weight gains of the female.

Part of this effect is undoubtedly attributable to the increasing proportion of dead embryos in the largest litters: these would weigh considerably less than their live litter-mates. It might also be argued that the correction for 7-day maternal weight, on which Text-figs. 2 and 8 are based, might result in an unreal picture of the situation. The weight gains were therefore retabulated, without correction, in terms of the number of *live* 18-day embryos. The discrepancy was not entirely made good.

The possibility remains that small immature females with large litters consume a proportion of their own body substance during pregnancy; in other words, that in their case extra-uterine weight gain is negative. This would be in line with our experience. Immature superpregnant females, in contrast to adult superpregnant females, appeared by day 18 of pregnancy to have exhausted all their fat reserves. Even such small deposits as the periovarian fat pad could no longer be seen. Pl. 4 shows a photograph of an immature female on day 18 of pregnancy. This female was 24 days old at pairing, and contained 22 implants, of which 16 were present as live embryos on day 18. The emaciation of this animal is in part obscured by her large girth, but is clearly seen in the region of the flanks.



Text-fig. 8. Average weight gains, for different numbers of embryos surviving to day 13, of treated and control TO females between days 7 and 18 of pregnancy, adjusted to a standard maternal 7-day weight. The lightly stippled areas show the gains per embryo surviving to day 13. Number above each column denotes number of females in group.

SUMMARY

1. Weight increase of female mice during pregnancy was studied in normal pregnancies, and also in females of various ages in which superovulation and mating had been induced by gonadotrophin treatment. In some spontaneous and induced pregnancies, the weights of the gravid uterus and of the live embryos were taken at autopsy $18\frac{1}{2}$ days *post coitum* in addition to the total weight of the intact pregnant female.

2. The main weight increase in both treated and control pregnancies occurred between about $10\frac{1}{2}$ and $16\frac{1}{2}$ days *post coitum*. The weight gain per embryo showed a linear decrease with increasing numbers of embryos, and a linear increase with the

$7\frac{1}{2}$-day weight of the female. When the data were adjusted to a standard $7\frac{1}{2}$-day maternal weight, the total weight gain at first rose with embryo number, but declined when the number of embryos increased above the normal range.

3. The extra-uterine weight increase of the female declined with increasing numbers of embryos. We conclude that female mice carrying abnormally large numbers of embryos economized not only on embryonic growth but also on extra-uterine weight increase. Young mice carrying large litters appeared to use up a large proportion of their fat reserves during pregnancy.

REFERENCES

CHESLEY, L. C. (1944). Weight changes and water balance in normal and toxic pregnancy. *Amer. J. Obstet. Gynec.* **48**, 565–93.

ENGLE, E. T. (1927). Pregnancy following superovulation in the mouse. *Proc. Soc. Exp. Biol., N.Y.* **25**, 84–5.

FEKETE, E. (1954). Gain in weight of pregnant mice in relation to litter size. *J. Hered.* **45**, 88–9.

FALCONER, D. S. (1955). Patterns of response in selection experiments with mice. *Cold Spr. Harb. Symp. Quant. Biol.* **20**, 178–96.

FOWLER, R. E. & EDWARDS, R. G. (1957). Induction of superovulation and pregnancy in mature mice by gonadotrophins. *J. Endocrin.* **15**, 374–84.

HEALY, M. J. R., McLAREN, A. & MICHIE, D. (1959). Superpregnancy in the mouse. 3. Foetal growth. In preparation.

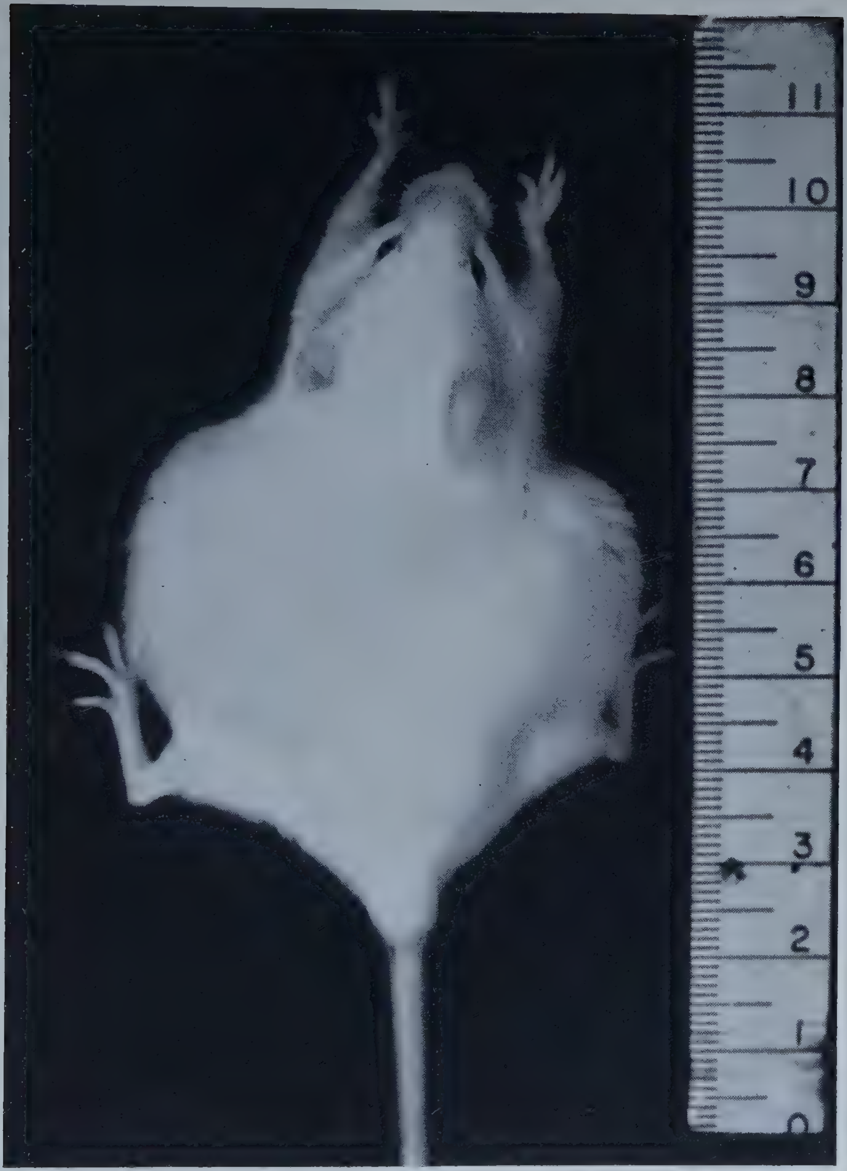
McLAREN, A. & MICHIE, D. (1959). Superpregnancy in the mouse. 1. Implantation and foetal mortality after induced superovulation in females of various ages. *J. Exp. Biol.* **36**, 281–300.

RUNNER, M. N. (1950). Induced ovulations in immature mice as a source of material for studies on mammalian eggs. *Anat. Rec.* **106**, 313–14 (Abstr.).

STANDER, H. J. & PASTORE, J. B. (1940). Weight changes during pregnancy and puerperium. *Amer. J. Obstet. Gynec.* **39**, 928–37.

EXPLANATION OF PLATE

An immature superpregnant TO female mouse, photographed $18\frac{1}{2}$ days *post coitum*. Note the signs of emaciation in the region of the flanks.



ANNE McLAREN AND DONALD MICHIE—SUPERPREGNANCY IN THE MOUSE

(*Facing p.* 314)

THE PHYSICAL PROPERTIES OF THE SWIMBLADDER IN INTACT CYPRINIFORMES

By R. McN. ALEXANDER
*Department of Zoology, University of Cambridge**

(*Received* 22 *October* 1958)

INTRODUCTION

The functions of the swimbladder make its physical properties important.

In the first place, to be hydrostatically adequate, the volume of the swimbladder must be such that the specific gravity of the fish is equal to that of its medium.

Further, it is desirable for a fish that the rate at which its buoyancy changes with pressure as it moves away from the depth at which it has neutral buoyancy should be as small as possible. The less the rate of change of buoyancy with depth, the less the effort which the fish must exert to maintain its station at a given distance from the equilibrium depth. Since the secretion and resorption of gas are slow processes, and the 'Gasspuckreflex'† of physostomes is irreversible except at the surface, restriction of the rate of change of buoyancy with depth by mechanical means will be useful. Such restriction could be achieved for depths less than the equilibrium depth by the possession of a highly inextensible swimbladder wall. This would confer no advantage at greater depths unless it was also rigid. The ossified swimbladder of the fossil Coelacanthi (see Williamson, 1849; Woodward, 1909) was presumably fairly rigid, but no comparable structure is known in modern fish (the swimbladder of *Latimeria*, if it exists at all, is rudimentary and unossified (Millot, 1954)). A swimbladder with a wall of limited extensibility, and with an internal pressure which, at the equilibrium depth, exceeded the external pressure would, however, change its volume with depth less markedly than a bubble of free air, at greater as well as at lesser depths. This point will be demonstrated later in the derivation of equation (5), which is applicable equally to small increases and to small decreases of pressure.

Where a sensory function has to be considered, inextensibility will reduce sensitivity, and a compromise must be reached with the requirements of the hydrostatic function. The operation of the Weberian ossicles will require a certain tautness in the swimbladder wall, such as might be obtained by the maintenance of excess pressure in the swimbladder. In this case sensory and hydrostatic requirements coincide, though an unnecessarily high pressure would reduce sensory sensitivity.

Evans & Damant (1928), using a hypodermic needle attached to a manometer, found that the roach maintains an excess pressure averaging 6 cm. Hg in its swimbladder. Franz (1937) claimed to find a slightly larger pressure in the minnow. Apparently the excess pressure discussed hypothetically in the above paragraphs does in fact occur in at least some Cypriniformes.

---

* Now at the Department of Zoology, University College of North Wales, Bangor.

† This term, literally 'gas-spitting reflex', is used by German authors and by Fänge (1953) to denote the release of gas through the pneumatic duct.

This paper is concerned with a method whereby the physical properties whose importance has been shown above have been determined for the swimbladders of a variety of Cypriniformes. These properties are volume, excess internal pressure, extensibility and rate of change of swimbladder volume with external pressure; this last property is expressed by a quantity which I call relative sensitivity.

DEFINITIONS

The quantities by which the physical properties of the swimbladder are expressed require a definition. They are listed below.

(1) The *percentage volume* of the swimbladder is the ratio between the volume of the swimbladder gas in millilitres at the depth to which the fish is adapted and the weight of the fish in grammes, expressed as a percentage. For reasons of convenience I follow Evans & Damant (1928) and Black (1948) in using the weight rather than the volume of the fish. My values must be multiplied by the specific gravity of the fish to obtain values exactly comparable with those of Akita (1936), Plattner (1941) and Jones (1951) who compare the volume of the swimbladder to that of the fish. The difference is in any case small.

(2) The *relative sensitivity* of the swimbladder is the ratio of the amount by which its volume changes when the external pressure is increased by a small amount from that to which the fish is adapted, to the amount by which the initially equal volume of a free bubble of air changes when exposed to the same increase of pressure. It has been found convenient to use pressure increases of 2 cm. Hg.

(3) The *excess internal pressure* of a swimbladder is the amount by which the pressure of the gases in the swimbladder exceeds that of the water surrounding the fish, at the depth to which the fish is adapted. The term 'excess pressure' is less rigidly used; it refers to the amount by which the pressure of the gases in the swimbladder exceeds that of the water surrounding the fish under the conditions stated in the context.

(4) The *extensibility* of a swimbladder is its increase in volume per cm. Hg increase in excess pressure, given as a percentage of the volume at 2 cm. Hg excess pressure. As it often varies with excess pressure, the excess pressure or range of excess pressures for which it is determined should be stated. Its value at the excess internal pressure is the *effective extensibility*.

MATERIAL

Experiments on the following species will be reported in this paper:

Cyprinoidei:	*Leuciscus leuciscus* (L.)	Dace
	Rutilus rutilus (L.)	Roach
	Scardinius erythrophthalmus (L.)	Rudd
	Phoxinus phoxinus (L.)	Minnow

Cyprinoidei:	*Tinca tinca* (L.)	Tench
	Gobio gobio (L.)	Gudgeon
	Abramis brama (L.)	Bream
	Alburnus alburnus (L.)	Bleak
	Cyprinus carpio L.	Carp
	Carassius carassius (L.)	Crucian carp
	C. auratus (L.)	Goldfish
Siluroidei:	*Amiurus nebulosus* (Lesueur)	

METHOD

Since the effective physical properties of the swimbladder may be affected by muscular tonus and the stiffness of the body wall, it is desirable that they should as far as possible be determined on intact living fish. The measurements were therefore made on intact, unoperated fish in a state of light anaesthesia.

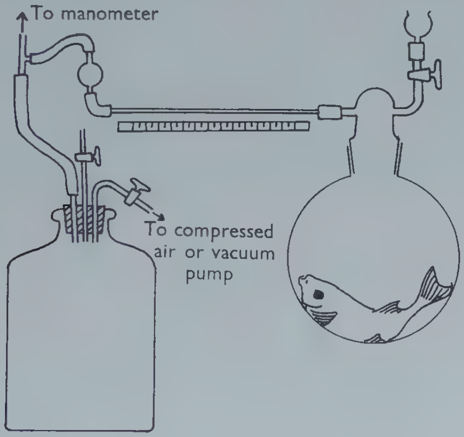


Fig. 1. The apparatus.

Principle

A fish is subjected to changes of pressure. The corresponding changes in the volume of its swimbladder gases are determined: (1) In the swimbladder in the intact fish; (2) when the restraining influence of the swimbladder wall has been eliminated.

From the results it is possible to calculate the volume, relative sensitivity, excess internal pressure and extensibility of the swimbladder.

Apparatus

The apparatus is shown in Fig. 1. All the rubber tubing indicated is pressure tubing, and this was made fast by wiring wherever this seemed advisable. A spring-loaded canvas sling was used to retain the dome on the flask at high pressures, and the joint was only very slightly greased to ensure a firm grip. The bulb between the capillary and the manometer eliminates the possibility of water being sucked back into the manometer at reduced pressures.

This apparatus was constructed in three sizes, whose dimensions are given in Table 1.

Table 1. *Dimensions of the apparatus*

Reference letter ...	Q	q	qq
Capacity of flask (ml.)*	5000	250	100
Neck size of flask*	B 55	B 34	B 24
Capacity of capillary (ml./cm.)†	0·078	0·0270 and 0·0224	0·00414
Weights of fish suitable (g.)	50–120	15–40	2–15

* Manufacturer's specifications (Quickfit and Quartz Ltd.).

† Determined by weighing the water which fills a measured length of capillary.

The domes of the q and qq apparatus were fitted on their sides with knobs for convenience in handling.

The flask was filled with an aqueous solution containing about 0·3 % urethane. The exact concentration used depended on the species of fish under investigation. To avoid bubble formation at low pressures the solution was made up with water of reduced air content: water taken from a geyser at about 55° C. and allowed to cool to room temperature in a brim-full flask covered with a Petri dish was found suitable.

A solution of about 0·7 % urethane was used as the initial anaesthetic.

Experimental method

Part I. The fish was kept for at least 24 hr. for adaptation before use in an aquarium containing water to a depth of 28 cm. It was moved directly from this to the stronger anaesthetic solution, in which it was kept for 10–20 min. until spontaneous swimming movements had ceased, but respiratory movements remained normal. This degree of anaesthesia persisted in the more dilute urethane in the apparatus, and was necessary to prevent the fish from swimming up and down in the flask, thus altering the pressure on its swimbladder, and to discourage the 'Gasspuckreflex', which can be released by fright. If the fish released bubbles in the anaesthetic it was returned to the aquarium for at least 24 hr. for re-equilibration.

The anaesthetized fish was put into the urethane solution in the flask. Its mouth was there inspected, and any bubbles of air in it removed. The dome was replaced firmly, and the spring-loaded sling attached. The funnel was used to fill the flask completely with the dilute urethane solution, excluding all bubbles of air. The flask was then attached to the rest of the apparatus and the funnel used to pass solution in until it extended along the capillary to form a meniscus at a convenient position. The rubber tubing connecting the flask to the capillary was wired to prevent leakage. It is very important that the apparatus should include no air bubbles in the flask or behind the meniscus.

The fish usually sank to the bottom of the flask. In this position in the Q, q or qq apparatus it would be 28, 15 or 10 cm., respectively, below the level of the capillary. When the manometer read zero it was thus at a pressure about 0, 1 or 1·5 cm. Hg less than that occurring at the bottom of the aquarium. This information will be used in determining the excess internal pressure of the swimbladder, the bottom of the aquarium being taken, for convenience, as the depth to which the fish was adapted. It was not thought advisable to define the depth to which it was adapted more precisely by using a shallower aquarium or a horizontally divided one, as such conditions would minimize the importance of buoyancy adjustment, which might well fail to occur (see Meesters & Nagel, 1934; Copeland, 1952).

Occasionally, mainly in work at reduced pressures, the fish floated. As the pressure at the top of the flask is lower than at the bottom it was necessary to subtract this difference from the manometer readings for the pressures at which the fish floated.

The pressure was then increased by stages to +24 cm. Hg* (manometer reading), and the position of the meniscus was observed at each pressure. It receded due to compression of the swimbladder and distortion of the apparatus. (The latter was determined in blank experiments, found to be very nearly constant for each apparatus, and corrected for.) The tap to the atmosphere was then opened to release the pressure and the fish was left for 15–20 min. for recovery before repetition of the measurements, or measurements at reduced pressure. In general, measurements were repeatable provided that such an interval was allowed between runs. The interval was apparently made necessary by a small 'slow elastic' effect in the swimbladder wall. Occasionally, marked differences were found between readings. When the displacement of the meniscus on increasing the applied pressure from 0 to 24 cm. Hg differed in two sets of readings by more than 0·5 cm., a significant change was deemed to have occurred and only the first set of readings was used. In other cases means were taken. This phase of the experiment was abandoned immediately if any gas bubbles were released by the fish.

Part II. The pressure was reduced strongly (usually to 36 cm. Hg below atmospheric) to extract a quantity of gas from the swimbladder. This process is of course only effective with physostomes, for which alone this technique is designed. The gas passed to the dome of the flask and atmospheric pressure was restored. Determinations of meniscus position were then made at the same pressures as before. Successive sets of readings could now be made without any interval and were found to agree well with one another. If sufficient gas had been extracted, the wall of the swimbladder would now be slack and the swimbladder gas, whether in the swimbladder or in the dome, would be unconstrained by it. To ensure that this was the case a stronger reduction of pressure (usually to 46 cm. Hg below atmospheric) was applied to extract more gas, and the measurements were repeated. This check was made over a range of pressures including the lowest used for previous readings, since it is at the lowest external pressures that the swimbladder wall is most likely to be taut. If the new measurements agreed with the previous series, the gas had clearly been unconstrained in both cases. The volume of the gas would then, by Boyle's Law, be inversely proportional to the external pressure.

---

* +18 cm. Hg in the first few experiments.

The animal was then taken out of the apparatus, killed, dried with a towel and weighed. The experiment was finished.

The bubbles released at decompression are not retained at the same depth as the fish, but rise into the dome. In the largest (Q) apparatus they will there always be subjected to a pressure about 2 cm. Hg less than that on the fish. Hence when the total pressure on the fish changes from 78 to $(78+\Delta P)$ cm. Hg (i.e. by $100\Delta P/78$ %) the total pressure on these bubbles will change from 76 to $(76+\Delta P)$ cm. Hg (i.e. by $100\Delta P/76$ %). The latter percentage pressure change is $100(78/76-1)$ or about 2·6 % larger than the former. The bubbles released on decompression to −36 cm. Hg probably did not exceed one-third of the swimbladder gas. The displacements observed would differ by less than 1 % from those to be expected if all the gas had remained at the level of the fish. The error would be correspondingly less in the smaller apparatus.

The meniscus position is necessarily very sensitive to temperature change. A change of 0·1° C. in the temperature of the liquid contents of the largest flask would move the meniscus about 1 cm. Temperature fluctuations were found, however, to be slow. The meniscus might move appreciably in the 20 min. intervals between sets of readings, but the movement was too slow to be significant in the 2 min. period required to take a set of readings. The small thermal capacity of the smallest (qq) flask, containing only about 100 ml. of solution, made it undesirable to leave water to evaporate from its surface, or to handle it more than necessary.

Method of calculation

The differences between the meniscus positions for +18 cm. Hg and +24 cm. Hg in Parts I and II of the experiment were found to be identical or nearly so in most cases (except in the case of the bream). Thus the swimbladder wall was slack over this range of pressures even before decompression and removal of gas. This being so, the +24 cm. Hg readings of Parts I and II represented identical gas volumes, and it was possible to use them as points of reference for superposition of the curves of volume change (shown as meniscus displacement) as is done in Fig. 2. The meniscus positions representing these identical volumes were not in fact often identical, owing to temperature changes, retention of drops of water in the bulb after decompression, and the slight expansion of released bubbles as they rose to the dome.

The information required can be obtained from these curves as described below.

(1) *Percentage volume.* The volumetric capacity of the capillary had been determined. The extent of the volume changes occurring in the course of the experiment was, therefore, known. From those occurring in Part II it was possible to calculate the volume of gas present at, for instance, manometer zero by applying Boyle's Law. The volume of the gas in the swimbladder in Part I was less than this by the volume represented by the difference between the intercepts of the two curves on the displacement axis (see Fig. 2). Since the fish had been weighed, the percentage volume of the swimbladder could be calculated.

(2) *Relative sensitivity.* The displacements of the meniscus on increasing the pressure from manometer zero to +2 cm. Hg in parts I and II are known. As the volume of the gas at manometer zero is slightly greater in Part II than in Part I, the ratio of displacements must be multiplied by this ratio of volumes to obtain the relative sensitivity.

(3) *Excess internal pressure.* The total pressure in the swimbladder when a given pressure is applied to the fish in Part I is equal to the total pressure required in Part II to adjust the volume of the swimbladder gas to the same value. It is thus possible to read off the excess pressure of the swimbladder at any point on the Part I curve as the difference between the abscissae of the two curves at the relevant ordinate. That corresponding to the pressure at the bottom of the aquarium was the excess internal pressure.

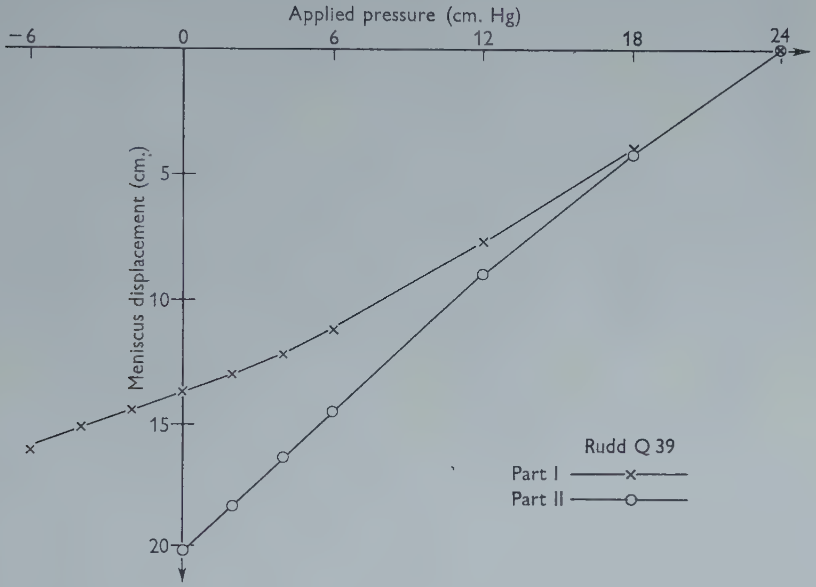


Fig. 2. Graphs of corrected meniscus displacement against applied pressure (in cm. Hg above manometer zero) for parts I and II of the experiment on the rudd Q 39.

The identity of the excess internal pressures in the two sacs of cyprinoid swimbladders is assumed. The following evidence supports this assumption:

(i) Evans & Damant (1928) found a pressure difference greater than 1 cm. Hg between the two sacs of only four roach out of seventeen. Their measurements appear, for the most part, to have been made to the nearest cm. Hg.

(ii) In another series of experiments, involving a variety of cyprinoid species, the sphincter between the two sacs of dead fish was never found to be closed except after removal of the tunica externa. In this operation the tunica externa was pulled round the sphincter and may have constricted it.

On the other hand, Wittenberg (1958) states, without giving evidence, that the ductus communicans of the goldfish is open only during secretion.

(4) *Extensibility*. From the various volumes of the swimbladder in Part I and from the corresponding excess pressures in it a curve of swimbladder extension against excess pressure can be constructed, as is done in Fig. 3 for the four rudd examined. As the volume of a swimbladder at zero excess pressure is indeterminate, and the volume at which its wall just becomes taut is not accurately determinable on account of the high extensibility found at low pressures (see later), the extension is calculated as the percentage difference from the volume with 2 cm. Hg excess pressure.

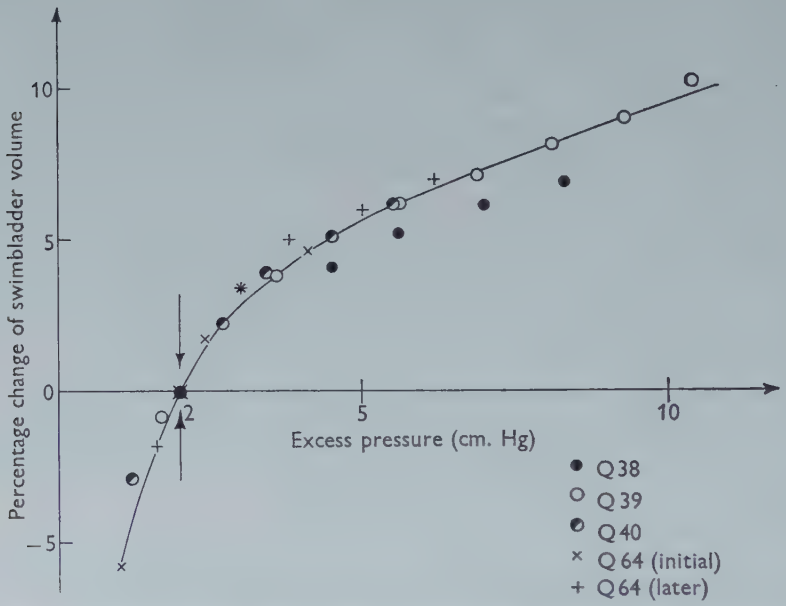


Fig. 3. Extension plotted against excess pressure for the swimbladders of the four rudd examined.

The bream swimbladder normally has a higher excess internal pressure than is found in other species. The differences between the meniscus positions at +18 cm. Hg and +24 cm. Hg in Parts I and II were not for it identical. This means that the swimbladder wall is not slack at +18 cm. Hg applied pressure. As it is in general slack at this pressure in all other species examined, it seems reasonable to assume that it will be slack by +24 cm. Hg in the bream, and as the apparatus was not suitable for work at higher pressures the values for +24 cm. Hg were superimposed as in other species. It remains possible that the values for excess pressure in the bream may be slightly too low.

RESULTS

The results of these experiments are given for individual fish in Appendix 2. They are summarized and compared with the data of previous workers below.

(1) *Percentage volume*. There is considerable variation of swimbladder volume within each species, but the variation between species is larger. There is a clear

distinction, for instance, between the small swimbladders of carp (5·0–6·5%), the medium ones of bream (7·0–8·2%) and the large ones of roach (8·8–10·4%).

Mean values for each species, with those of previous authors, are given in Table 2.

Table 2. *Percentage volumes of the swimbladders of Cypriniformes*

Species	Mean % volume	Previous values
Roach	9·9	9·74* (Plattner, 1941) 9·1 (Evans & Damant, 1928)
Rudd	8·9	10·61 (Plattner, 1941)
Dace	8·0	—
Minnow	6·1	6·6† (calculated from data in Franz, 1937)
Tench	7·0	7·74 (Plattner, 1941)
Bream	7·6	—
Bleak	6·7	—
Carp	5·8	—
Goldfish	7·9	8·25 (Plattner, 1941)
Crucian carp	8·0	—
Gudgeon	6·2	—
Amiurus	5·8	—

* Plattner calculated his mean incorrectly. The correct value is given here.
† A highly aberrant value of 9·2% is neglected.

Table 3. *Relative sensitivities of the swimbladders of Cypriniformes*

Species	Mean relative sensitivity	Species	Mean relative sensitivity
Roach	0·41	Bleak	0·64
Rudd	0·44	Carp	0·72
Dace	0·65	Goldfish	0·66
Minnow	0·79	Crucian carp	0·57
Tench	0·50	Gudgeon	0·73
Bream	0·23	*Amiurus*	0·87

The results agree reasonably well with those of previous authors, except in the case of the rudd. It should be noted that, whereas my values are for the swimbladder gas only, Plattner's values for cyprinoids include the swimbladder wall.

(2) *Relative sensitivity.* Considerable variation within each species is again exceeded by variation between species. The values of 0·18–0·26 for the bream contrast strongly with those of 0·63–0·93 for the carp.

The mean values found for each species are given in Table 3.

(3) *Excess internal pressure.* A much lower pressure is found in *Amiurus* than in any of the Cyprinidae examined. Within the Cyprinidae themselves there is a wide range of variation between the extremes of the gudgeon (1·6–2·9 cm. Hg) and the bream (8·9–14·6). Most of them have mean excess internal pressures between 2·0 and 3·1 cm. Hg; only the roach, rudd and bream lie outside this range, all with much higher pressures.

The mean values are given in Table 4 with those of previous authors.

(4) *Extensibility.* Extension is plotted against excess pressure for the four rudd which were examined in Fig. 3. It will be seen that there is good agreement between the individuals of the species. This is also true of the other species examined.

Table 4. *Excess internal pressures of the swimbladders of Cypriniformes*

Species	Mean excess internal pressure (cm. Hg)	Previous values		
		Anterior sac	Posterior sac	Authors
Roach	7·0	5·9	6·2	Evans & Damant (1928)
Rudd	6·1	—	—	—
Dace	2·7	—	—	—
Minnow	2·6	7·8*	6·8*	Franz (1937)
Tench	2·9	—	—	—
Bream	10·8	—	—	—
Bleak	3·1	—	—	—
Carp	2·6	—	—	—
Goldfish	2·4	—	—	—
Crucian carp	3·0	—	—	—
Gudgeon	2·1	—	—	—
Amiurus	0·6	—	—	—

* The serious discrepancy between Franz's values and mine is discussed in Appendix 1.

The effective extensibilities of these species, being the gradients of these curves at the relevant mean excess internal pressures, are given in Table 5. There are no comparable data in the literature. No value was obtained for *Amiurus*, whose excess pressure was less than 2·0 cm. Hg at the lowest pressures at which readings were taken.

Table 5. *The effective extensibilities of the swimbladders of Cypriniformes*

Species	Effective extensibility (%/cm. Hg)	Species	Effective extensibility (%/cm. Hg)
Roach	1·0	Bleak	2·7
Rudd	0·9	Carp	3·5
Dace	3·9	Goldfish	3·3
Minnow	4·1	Crucian carp	1·2
Tench	1·1	Gudgeon	4·7
Bream	0·4		

In Figs. 4–6 extensibility—the gradient of the extension curve—is plotted against excess pressure. The extensibilities of the swimbladders of roach, rudd, tench, goldfish, crucian carp and possibly minnow and gudgeon agree in falling from a high level to a steady one of about 1%/cm. Hg as the pressure rises. This fall is completed at 3–6 cm. Hg excess pressure. The bream swimbladder is less extensible than the others throughout, while the extensibilities of dace and carp are asymptotic to values of about 2·5%/cm. Hg, and of bleak to just under 2%/cm. Hg.

The excess internal pressure is extremely important in determining the effective extensibility. Thus the goldfish and the rudd have closely similar curves of extensibility against excess pressure, but while the former has an excess internal pressure of 2·4 cm. Hg and an effective extensibility of 3·3%/cm. Hg, the latter has 6·1 and 0·9.

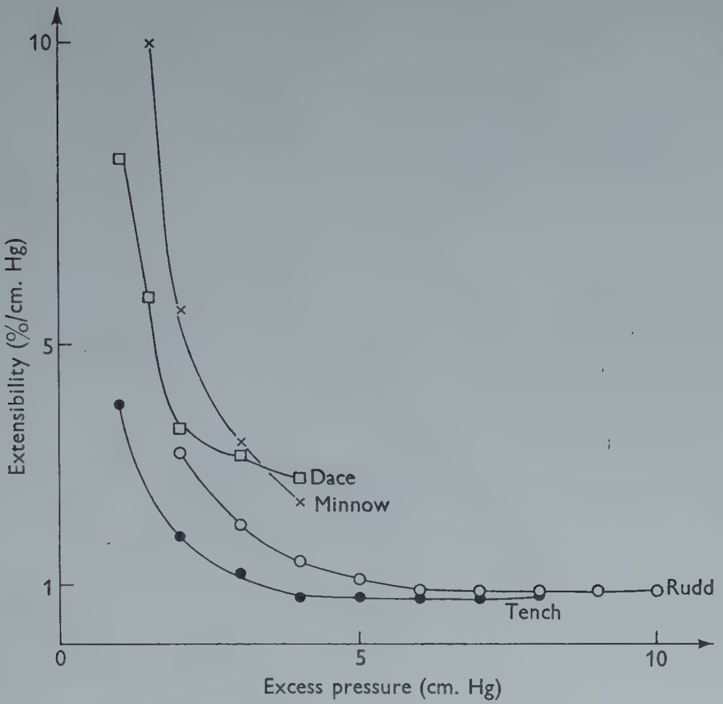


Fig. 4. The relation between extensibility and excess pressure for the swimbladders of dace, minnow, tench and rudd.

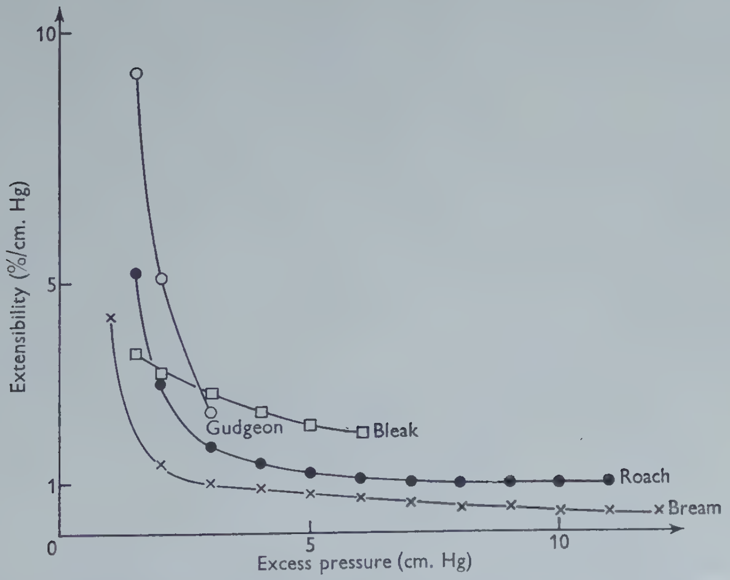


Fig. 5. The relation between extensibility and excess pressure for the swimbladders of bleak, gudgeon, roach and bream.

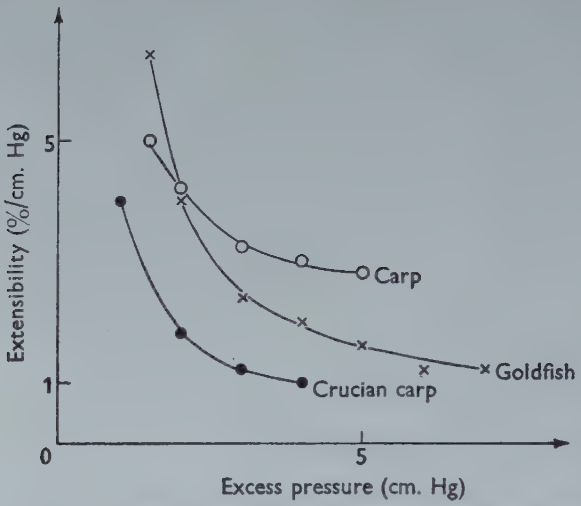


Fig. 6. The relation between extensibility and excess pressure for the swimbladders of carp, goldfish and crucian carp.

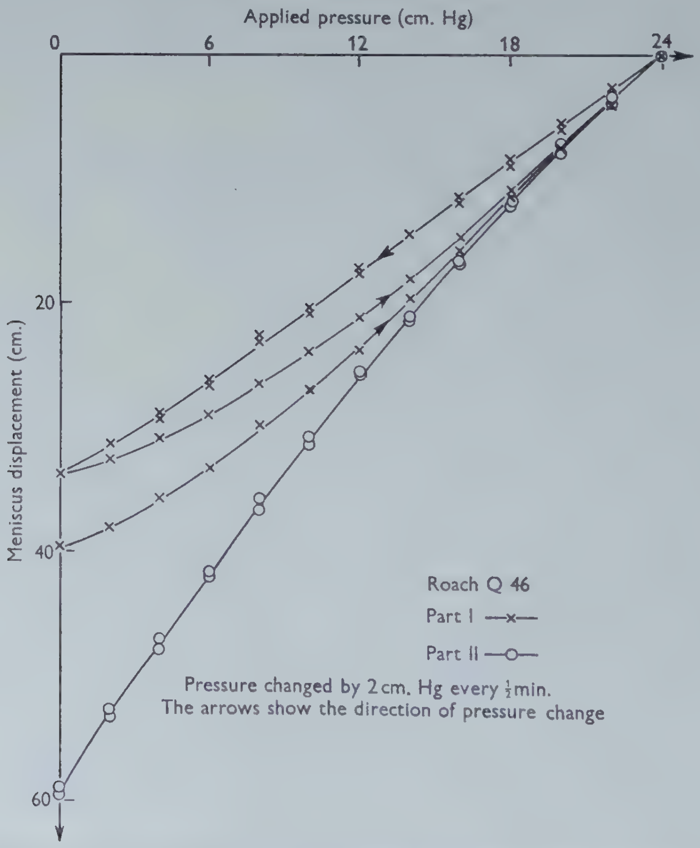


Fig. 7. Meniscus displacements for a roach undergoing cyclical pressure changes, showing a hysteresis effect due to the slow elastic behaviour of the swimbladder.

The variability of extensibility with pressure, even over small ranges of extension, requires explanation in terms compatible with Hooke's Law. It seems probable that as the swimbladder extends, its elastic elements do not all become taut at once. The final value of the extensibility is reached when all these elements are taut: the higher values at lower pressures are due to the slack condition of some of the elements.

There is no evidence to identify these elements which are supposed to tauten successively. They may be molecular chains (see King & Lawton, 1950) or they may be fibres.

The slow elastic effect

The slow elastic behaviour of the swimbladder has already been mentioned. On account of it, when a cyprinoid is exposed to cyclical changes of pressure its volume changes show a hysteresis effect. This is illustrated in Fig. 7, which is a graph of the same type as Fig. 2. A roach in the Q apparatus underwent cyclical changes of pressure between manometer zero and +24 cm. Hg. The pressure was changed by 2 cm. Hg every 30 sec. The first two cycles are shown. The meniscus movements showed a marked hysteresis. In Part II of the experiment the readings were obtained on a similar pressure cycle. There is no observable hysteresis. The hysteresis found in Part I is thus a property of the fish, not of the apparatus.

Franz (1937) and Moehres (1940) have shown that the 'Gasspuckreflex' in the minnow and the gudgeon, respectively, has a lower threshold for a slow than for a fast pressure drop. Moehres found mean thresholds for the gudgeon of 6·22 cm. Hg when the pressure was reduced at 50 cm. Hg/min. and 2·17 cm. Hg for 2·4 cm. Hg/min. Three explanations of this are possible.

(1) The threshold was at 2 cm. Hg but there was a delay of 5 sec. between the passing of the threshold and the response.

(2) Owing to a slow elastic effect the expansion of the swimbladder during a given reduction in pressure is greater if the reduction is slow than if it is fast. This assumes, as seems probable, that the necessary stimulus for the 'Gasspuckreflex' is the expansion of the swimbladder to a definite size.

(3) There is both a delay and a slow elastic effect.

As a slow elastic effect has been demonstrated and the assumption involved in (2) appears to be justified, explanation (2) or (3) must be accepted. As the passage of air from the swimbladder to and out of the mouth cannot be instantaneous, we are driven to explanation (3).

It is concluded, then, that the dependence of the apparent 'Gasspuckreflex' threshold on the rate of reduction of pressure must be explained in terms of a delayed response and the slow elastic effect. Insufficient work has been done for it to be possible to estimate the relative importance of these two factors.

DISCUSSION

(1) *Factors controlling relative sensitivity*

The following discussion concerns the relationship of relative sensitivity to extensibility and excess internal pressure.

Consider a closed elastic gas-filled bladder and let its volume be v and the excess pressure in it p at external pressure P.

Now let the external pressure increase by a small amount δP. The excess pressure is now, say $(p+\delta p)$ and the total internal pressure $(P+\delta P+p+\delta p)$. The volume is now $v(1+\delta pe)$, where e is the extensibility of the bladder at excess pressure p. The change in volume

$$\delta v = v\delta pe. \tag{1}$$

By Boyle's Law, since the mass of gas in the bladder is unchanged

$$\left.\begin{aligned}(P+p)v &= (P+\delta P+p+\delta p)\, v(1+\delta pe),\\ P+p &= P+\delta P+p+\delta pe(1/e+P+\delta P+p+\delta p),\\ \delta P &= -\delta pe((1/e)+P+\delta P+p+\delta p),\end{aligned}\right\} \tag{2}$$

From (1) and (2)

$$\delta v = \frac{-\delta P v}{(1/e)+P+\delta P+p+\delta p}. \tag{3}$$

Now consider an unconstrained mass of gas also having volume v at pressure P. When the pressure rises to $(P+\delta P)$ the volume becomes $Pv/(P+\delta P)$. Whence the change in volume

$$\delta v' = \frac{\delta P v}{P+\delta P}. \tag{4}$$

From (3) and (4)

$$\frac{\delta v}{\delta v'} = \frac{P+\delta P}{(1/e)+P+\delta P+p+\delta p}. \tag{5}$$

Now consider the bladder as the swimbladder of a fish adapted to an external pressure P. $\delta v/\delta v'$ is the relative sensitivity, p is the excess internal pressure and e is $\frac{1}{100}$ of the effective extensibility.

If we apply equation (5) to the fish of my experiments we may write, taking cm. Hg as the unit of pressure, $P \simeq 78$, $\delta P = 2$, $e < 0{\cdot}05$. δp cannot exceed 2 and can be ignored as it is small compared to $((1/e)+P)$.

We may thus re-write equation (5):

$$\text{relative sensitivity} \simeq \frac{80}{80+(1/e)+p}. \tag{6}$$

This expression gives values for relative sensitivity which agree well with those calculated by the normal method.

In equation (6) $1/e$ varies from 21 (gudgeon) to 250 (bream) and is extremely important in determining relative sensitivity. p varies among Cyprinidae from 2·1 (gudgeon) to 10·8 (bream) and is of relatively little importance. But e varies markedly with p, being higher at low values of p. Excess internal pressure thus influences relative sensitivity almost entirely through its effect on extensibility.

Although its relationship to the other properties which have been measured makes the inclusion of relative sensitivity tautologous, it is convenient to retain it as it summarizes the effect on the swimbladder of small pressure changes.

(2) *The rate of change of buoyancy with depth*

It has been shown that all the species examined have an excess pressure in the swimbladder. It was suggested in the introduction that such a pressure would be necessary to the proper functioning of the Weberian ossicles. The mean excess internal pressure is found to be 0·6 cm. Hg for *Amiurus*, and between 2 and 3 cm. Hg for most of the Cyprinidae examined. The roach, rudd and bream have much higher excess internal pressures (7·0, 6·1 and 10·8 cm. Hg, respectively). The effect of these is to reduce the effective extensibility and so the relative sensitivity of the swimbladder: their mean values for this last quantity are 0·41, 0·44 and 0·23, respectively. Such extreme increases in pressure and decreases in relative sensitivity can hardly be advantageous to the sensory functioning of the swimbladder, and we must assume that there is a tendency in the Cypriniformes to evolve for its own sake a low rate of change of buoyancy with depth, such as was discussed in the introduction. Such a property will only lead to a net saving of energy, and our assumption will only be valid, if the density of the fish is so close to that of its medium that the fish's plane of neutral buoyancy lies within its normal vertical range. A subsequent paper (Alexander, 1959) will present evidence that this is in fact the case.

SUMMARY

1. A method is described whereby certain physical properties of the swimbladder have been determined in intact, unoperated Cypriniformes. These properties are volume, excess internal pressure, extensibility, and relative sensitivity (an index of the rate of change of volume with depth).

2. The mean volume of the swimbladder varies in the twelve species examined from 5·8 to 9·9 ml./100 g. body weight.

3. In most Cyprinidae the swimbladder gases are maintained at a mean pressure 2–3 cm. Hg above that of the surrounding water at the depth to which the fish is adapted. Higher pressures occur in a few species, most notably the bream (10·8 cm. Hg). A much lower pressure is found in *Amiurus*. An excess internal pressure is probably necessary to provide a taut swimbladder wall to operate Weberian ossicles.

4. The swimbladder wall has non-linear elastic properties such that as the excess pressure in the swimbladder rises the extensibility of its wall falls. The swimbladder also shows slow-elastic properties. The significance of these properties is discussed.

5. Excess internal pressure and low extensibility combine to limit the rate at which the volume of the swimbladder, and so the buoyancy of the fish, changes with depth. For small depth changes the swimbladder of the bream (an extreme case) changes its volume only one-quarter as much as would a free air-bubble.

I wish to thank Dr K. E. Machin and Dr G. M. Hughes for much advice, and the Development Commission for financial support. Mr H. R. Klose has helped me to obtain and care for fish, some of which I received through the kindness of Mr N. MacKenzie or of Dr H. K. Mann and Miss Elizabeth Steel.

REFERENCES

Akita, Y. K. (1936). Studies on the physiology of swimbladder. *J. Fac. Sci. Tokyo Univ.* sect. 4, **4**, 111–35.

Alexander, R. McN. (1959). The densities of Cyprinidae. *J. Exp. Biol.* **36**, 333–40.

Black, V. S. (1948). Changes in density, weight, chloride and swimbladder gas in the killifish, *Fundulus heteroclitus*, in fresh water and sea water. *Biol. Bull., Wood's Hole*, **95**, 83–93.

Copeland, D. E. (1952). The stimulus of the swimbladder reflex in physoclistous teleosts. *J. Exp. Zool.* **120**, 203–12.

Evans, H. M. & Damant, G. C. C. (1928). Observations on the physiology of the swimbladder in cyprinoid fishes. *Brit. J. Exp. Biol.* **6**, 42–55.

Fänge, R. (1953). The mechanisms of gas transport in the euphysoclist swimbladder. *Acta physiol. scand.* **30**, Suppl. 110, pp. 133.

Franz, G. (1937). Über den Reflex des Gasspuckens bei Fischen und die Funktion des Weberschen Apparates. *Z. vergl. Physiol.* **25**, 193–238.

Jones, F. R. H. (1951). The swimbladder and the vertical movements of teleostean fishes. I. Physical factors. *J. Exp. Biol.* **28**, 553–66.

King, A. L. & Lawton, R. W. (1950). Elasticity of body tissues. In *Medical Physics*, ed. O. Glasser **2**, 303–16.

Meesters, A. & Nagel, F. G. P. (1934). Über Sekretion und Resorption in der Schwimmblase des Flussbarsches. *Z. vergl. Physiol.* **21**, 648–57.

Millot, J. (1954). Le troisième Coelacanthe. *Le Naturaliste Malgache*, 1e suppl., pp. 26.

Moehres, F. P. (1940). Untersuchungen über die Frage der Wahrnehmung von Druckunterschieden des Mediums. (Versuche an Bodenfischen.) *Z. vergl. Physiol.* **28**, 1–42.

Plattner, W.-A. (1941). Études sur la fonction hydrostatique de la vessie natatoire des poissons. *Rev. suisse Zool.* **48**, 201–338.

Williamson, W. C. (1849). On the microscopic structure of the scales and dermal teeth of some ganoid and placoid fish. *Phil. Trans.*, pp. 435–75.

Wittenberg, J. B. (1958). The secretion of inert gas into the swim-bladder of fish. *J. Gen. Physiol.* **41**, 783–804.

Woodward, A. S. (1909). *The Fossil Fishes of the English Chalk*, part V, pp. 153–84. Palaeontogr. Soc. (Monogr.).

APPENDIX 1

Criticism of Franz's determination of the excess internal pressure of the minnow

Franz (1937) used the direct manometric method of Evans & Damant (1928) to determine the excess internal pressure of minnows. He found values of about 1 cm. Hg, with a maximum of 1·5 cm. Hg.

He was dissatisfied with this method, and did some further experiments in which he measured the volume of the swimbladder gases in the isolated swimbladder and after release from it. These measurements, which were very rough, showed that the isolated swimbladders had a mean excess internal pressure of about 1·2 cm. Hg. Franz believed, however, that the excess internal pressure in the intact fish was considerably higher. He found that the flotation pressure for a minnow whose body cavity had been opened and swimbladder exposed averaged about 6 cm. Hg above the value for the intact animal and supposed this to be due to pressure exerted by the body wall. He added this difference to the values obtained for the isolated swimbladder, and so obtained his mean values of 7·8 cm. Hg for the anterior sac and 6·8 for the posterior.

As the values obtained for the excess internal pressure of the minnow by the method described in this paper were very much lower than those reported by Franz, his work on the change of flotation pressure when the swimbladder is exposed was repeated. It was found necessary to take great care to avoid catching air bubbles in the opened body cavity. Of six minnows tested, three showed no change of flotation pressure and three showed decreases (perhaps due to stray bubbles of gas in the body cavities of intact fish). In no case was an increase in flotation pressure found. It seems probable that Franz's result was due to capture of air-bubbles in the opened body cavity. He describes no precautions against this hazard.

For this reason I reject Franz's high values for the excess internal pressure of the minnow. His direct measurements are probably more reliable, though he himself rightly criticizes the direct method as likely to give low values.

APPENDIX 2

Details of results

Species	Specimen	Weight (g.)	Excess internal pressure (cm. Hg)	Relative sensitivity	% vol.
Roach	Q 13	135	5·0	0·48	10·3
	Q 14	—	9·5	0·34	—
	Q 15	100	6·9	0·40	10·2
	Q 16	165	8·8	0·31	10·4
	Q 17	100	4·6	0·53	8·8
	Mean		7·0	0·41	9·9
Rudd	Q 38	130	8·3	0·42	8·5
	Q 39	70	6·9	0·40	8·9
	Q 40	40	5·2	0·44	9·3
	Q 64	—	4·1	0·48	—
	Mean	—	6·1	0·44	8·9
Dace	Q 10	—	3·4	0·55	—
	Q 18	100	3·1	0·61	8·5
	Q 19	90	1·8	0·73	8·2
	Q 22	105	3·4	0·60	8·4
	Q 48	55	3·2	0·54	8·5
	qq 7	5·56	2·0	0·89	7·7
	qq 8	4·62	1·7	0·60	6·5
	Mean		2·7	0·65	8·0
Minnow	q 47–70	41 (24 fish)	2·2	0·77	5·1
	qq 12	2·35	2·3	0·62	6·6
	qq 14	3·00	2·5	—	5·9
	qq 15	2·91	1·9	0·88	7·7
	qq 16	2·11	3·5	0·65	5·9
	qq 17	2·49	3·3	0·89	5·7
	qq 18	3·21	2·2	0·92	5·9
	Mean		2·6	0·79	6·1
Tench	Q 4	—	1·8	—	—
	Q 7	—	1·5	0·65	—
	Q 8	—	2·7	0·47	—
	Q 9	—	3·4	0·39	—
	Q 96	73	5·9	0·28	6·4
	Q 97	102	2·9	0·50	7·4
	qq 21	20·8	2·4	0·73	7·3
	Mean		2·9	0·50	7·0

APPENDIX 2 (*continued*)

Species	Specimen	Weight (g.)	Excess internal pressure (cm. Hg)	Relative sensitivity	% vol.
Bream	Q 65	105	10·6	0·26	8·0
	Q 66	100	8·9	0·21	8·2
	Q 67	95	14·6	0·18	7·7
	Q 68	60	10·6	0·22	7·0
	Q 94 (P 24)	65	9·4	0·26	7·1
	Mean	—	10·8	0·23	7·6
Bleak	q 83	16·3	1·9	0·65	6·3
	q 84	20·8	5·6	0·58	6·9
	q 85	15·8	1·7	0·68	7·0
	Mean	—	3·1	0·64	6·7
Carp	Q 41	65	1·3	0·93	5·0
	Q 42*	75	1·0	0·86	5·1
	Q 43	60	1·9	0·71	6·0
	Q 44*	40	1·1	0·83	5·0
	Q 49*	50	3·4	0·71	6·4
	q 80	38	3·5	0·63	6·1
	q 81	37	5·0	0·48	6·4
	q 82	45	3·5	0·64	6·5
	Mean	—	2·6	0·72	5·8
Crucian carp	Q 90	90	1·5	0·75	5·6
	Q 91	120	3·8	0·49	7·8
	Q 92	75	2·0	0·62	8·0
	Q 93 (P 21)	75	4·8	0·42	10·7
	Mean	—	3·0	0·57	8·0
Goldfish	Q 24	35	1·8	0·82	8·9
	Q 25	35	3·7	0·51	8·9
	q 43	40	2·8	0·48	6·3
	q 45	26	1·6	0·85	7·3
	q 46	—	2·0	0·65	—
	Mean	—	2·4	0·66	7·9
Gudgeon	Q 74–89	180 (17 fish)	2·9	0·73	5·9
	qq 1	11·3	1·8	0·84	6·5
	qq 2	9·3	1·6	0·61	6·2
	Mean	—	2·1	0·73	6·2
Amiurus	qq 3	3·74	0·8	1·00	6·6
	qq 4	4·67	0·4	0·61	5·2
	qq 5	2·48	0·5	1·00	5·7
	Mean	—	0·6	0·87	5·8

* Mirror carp.

THE DENSITIES OF CYPRINIDAE

By R. McN. ALEXANDER

*Department of Zoology, University of Cambridge**

(*Received* 22 *October* 1958)

INTRODUCTION

The densities of fish will be expressed in this paper by the sinking factor, defined by Lowndes (1942) as 1000 times the ratio of the density of the fish to that of its medium.

The term 'flotation pressure' will be used in this paper to denote the amount by which the pressure at which a fish has neutral buoyancy exceeds that to which it is adapted.

In a previous paper (Alexander, 1959) a method was described whereby the mean percentage volume and relative sensitivity were determined for a number of species of Cypriniformes. If the mean flotation pressure were known for each species, their sinking factors could be calculated.

Consider first a fish whose swimbladder volume depends only on Boyle's Law, and is not controlled by the swimbladder wall. It is adapted to an external pressure P at which its swimbladder has volume v', and it just floats at $(P+\Delta P)$ (i.e. its flotation pressure is ΔP). At this pressure its sinking factor is 1000 and the volume of its swimbladder $(v'+\Delta v')$.

Then, by Boyle's Law,

$$\left.\begin{aligned} Pv' &= (P+\Delta P)(v'+\Delta v'), \\ \Delta v' &= -\frac{\Delta P}{P+\Delta P}v'. \end{aligned}\right\} \tag{1}$$

In the case of a fish whose swimbladder gas is constrained by the swimbladder wall, the change of volume, Δv, will be rather smaller, and we must write

$$\Delta v = -\frac{\Delta P}{P+\Delta P}v'\frac{\Delta v}{\Delta v'}. \tag{2}$$

For small values of ΔP, the ratio $\Delta v/\Delta v'$ is the relative sensitivity. In my experiments, with the possible exception of those on the gudgeon, the mean value of ΔP for each species is sufficiently small for it to be possible to assume that $\Delta v/\Delta v'$ agrees with the relative sensitivity to at least one significant figure.

Δv is the amount by which the volume of the swimbladder, and hence that of the fish, differs from the value for neutral buoyancy. If the total volume of the fish is V

---

* Now at the Department of Zoology, University College of North Wales, Bangor

the relative difference in the volume of the fish from the value for neutral buoyancy is then, from equation (2)

$$\frac{\Delta v}{V} = -\frac{\Delta P}{P+\Delta P}\frac{v'}{V}\frac{\Delta v}{\Delta v'}. \tag{3}$$

The quotient v'/V is, for a fish whose specific gravity is not far from unity, approximately equal to one-hundredth of the percentage volume of the swimbladder. The relative change in sinking factor, S, is given by

$$\frac{\Delta S}{S+\Delta S} = -\frac{\Delta v}{V}. \tag{4}$$

From (3) and (4)

$$\frac{\Delta S}{S+\Delta S} = \frac{\Delta P}{P+\Delta P}\frac{v'}{V}\frac{\Delta v}{\Delta v'}. \tag{5}$$

But

$$S+\Delta S = 1000.$$

Therefore the sinking factor of the fish, S, is given by

$$S = 1000\left(1-\frac{\Delta P}{P+\Delta P}\frac{v'}{V}\frac{\Delta v}{\Delta v'}\right). \tag{6}$$

METHOD

Each fish was anaesthetized and placed in urethane solution in the 'Q' flask, and its flotation pressure determined. A mean was taken for each species. This mean, together with equation (6) and the results reported in another paper (Alexander, 1959) sufficed for the calculation of the sinking factor.

The results are given in Table 1.

Table 1. *The sinking factors of Cyprinidae*

Species	Flotation pressure (cm. Hg above manometer zero)	Mean	Relative sensitivity	% Volume	S.F.	Previous values
Roach	−6, −7, −2, −4	−5	0·41	9·9	1003	1001 (Plattner, 1941)
Rudd	−6, −1, −13, −9, −3, −6, −3	−6	0·44	8·9	1003	1013 (Plattner, 1941)
Dace	+1, −2, −1, −1	−1	0·65	8·0	1001	—
Minnow	−9, +4, −2, −6, −3, −7, −7	−4	0·79	6·1	1002	—
Tench	−12, −7, +3, −10, −7	−7	0·50	7·0	1003	1007 (Popta, 1910), 1009* (Plattner, 1941)
Bleak	−1, 0, −1	−1	0·64	6·7	1001	—
Carp	−3, −5, −5, −2, −4, −12, +1, −1, −2	−4	0·72	5·8	1002	—
Gudgeon	−15, −18, −10	−14	0·73	6·2	1008	—

* Plattner's wrongly calculated mean density is here corrected.

All these species appear normally to be a little, but only a little, denser than water. The present experiments give lower sinking factors for rudd and tench than those found by Popta (1910) and Plattner (1941). Plattner determined density by measuring weight and volume directly, usually to three significant figures. In my work, and in Popta's, precision was obtained more easily by determining density indirectly by measuring the amount by which it differed from that of water. It is perhaps significant that the gudgeon, which is the strictest bottom-liver among these species (see Jenkins, 1925; Moehres, 1940) has the highest sinking factor. The difference is small, however, and I have noticed in aquaria that tench as well as gudgeon spend a great deal of time resting on the bottom. Very much higher sinking factors occur in many species from other groups (see Jones & Marshall, 1953).

Table 2. *The product of percentage volume and relative sensitivity*

	Mean % volume	Mean relative sensitivity	Product
Roach	9·9	0·41	4·1
Rudd	8·9	0·44	3·9
Dace	8·0	0·65	5·2
Minnow	6·1	0·79	4·8
Tench	7·0	0·50	3·5
Bream	7·6	0·23	1·7
Bleak	6·7	0·64	4·3
Carp	5·8	0·72	4·2
Goldfish	7·9	0·66	5·2
Crucian Carp	8·0	0·57	4·6
Gudgeon	6·2	0·73	4·5
Amiurus	5·8	0·87	5·0

DISCUSSION

All of the Cyprinidae examined, except the gudgeon, have sinking factors very close to 1000. In nature the level of neutral buoyancy will probably frequently lie within the vertical range inhabited by the fish. Changes of depth will have very significant effects on the buoyancy of the fish. It will be to the advantage of the fish that these effects be as small as possible. It has been shown in equation (5) that the relative change of sinking factor as a fish moves through a given small depth from the equilibrium level is approximately proportional to the product of the percentage volume and relative sensitivity of its swimbladder. Of two fish having neutral buoyancy at the same depth, that for which this product is greater will have to expend more energy to maintain its station at any other given depth. The values of this product for the species which have been studied are shown in Table 2.

The product of percentage volume and relative sensitivity lies between 3·5 and 5·2, except for the bream in which it is only 1·7. This constancy is surprising. It means that there is a tendency for species which require a large swimbladder to avoid the disadvantage of an unusually high rate of change of buoyancy with depth by having a lower relative sensitivity than do species with smaller swimbladders. A statistical test shows that this tendency is real (the correlation coefficient between mean percentage volume and mean relative sensitivity is $-0·68$, giving $P < 0·02$). The bream has a considerable advantage over the other species in constancy of density with depth. It would be interesting to know whether this is related to any peculiarity in the behaviour of the bream.

Bone and Fat

The percentage volume of the roach swimbladder is greater by 4·1 than that of the carp (Alexander, 1959). If the remaining tissues of these fish were of equal specific gravity we should expect the sinking factor of the carp to be greater than that of the roach by 41. It is in fact found to be 1002, while that of the roach is 1003. The lack of any appreciable difference in sinking factor, in spite of the difference in swimbladder volume, must be explained in terms of differences in the constitution of the tissues of the two species. It was thought likely that fat and bone would be important, as a large proportion of the former would tend to give a low sinking factor, of the latter a high one. It therefore seemed appropriate to determine the proportions of these components in a number of species.

The fish was killed, roughly dried, and weighed. It was then placed in a beaker of water and boiled lightly. It was skinned, and the skin, with any loose scales, put in one dish. The bones were removed and put in another; great care was taken to miss none of them, and they were stripped of flesh as far as could conveniently be done with forceps.

The fish had now been separated into three components: the skin and scales, the skeleton, and the remainder, which we may call flesh. The scales and skeleton were cleaned by leaving them for (usually) two days in hydrogen peroxide solution (about 20 vol.). They were then washed, dried and weighed. The flesh was dried at 110° C. and ground finely. Both it and the water in which the fish had been boiled were extracted with ether. The ether was evaporated from the resulting solution and the fatty residue weighed. This would include ether-soluble materials other than fats, but not the fats of the skin.* The values for fat content must therefore be regarded as approximate.

The results are given in Table 3. The proportions of the components are given as percentages of body weight.

Roach of widely different sizes were used to determine whether there is any marked variation of composition with size. It was concluded that there is not.

Jacquot & Creac'h (1950) give values between 0·4 and 4·0% for the fat content of the edible parts of various Cyprinidae, with one exceptional value of 9·0% for a carp in breeding condition. As these values refer to edible parts only they are not strictly comparable to mine. None of the fish I examined contained more than 2% fat.

We must consider how the differences in composition which have been found will affect the buoyancy of the fish. For this discussion the specific gravities of the components must be known.

Spector (1956) gives the specific gravities of four teleost oils as 0·925, 0·900, 0·903 and 0·905. The mean of these is about 0·91.

---

* It seems unlikely that much fat was lost with the skin. An entire carp which was dried and extracted gave fat content (1·5%) within the range of variation found in the skinned and boned specimens.

Table 3. *Composition of Cyprinidae*

Species	Weight of fish (g.)	Weight of skeleton (%)	Weight of scales (%)	Weight of skeleton + scales (%)	Weight of fat (%)
Roach	20	4·5	4·2	8·7	—
	39	3·8	3·9	7·7	0·7
	41	5·5	4·2	9·7	—
	117	4·1	4·3	8·4	—
	130	3·1	2·9	6·0	—
	Mean	4·2	3·9	8·1	0·7
Dace	33	3·3	2·3	5·6	1·7
	45	3·6	1·7	5·3	1·8
	Mean	3·5	2·0	5·5	1·8
Carp	37	5·2	2·5	7·7	0·8
	38	5·3	2·2	7·5	0·8
	53	4·6	2·5	7·1	—
	57	4·4	1·9	6·3	0·7
	60	4·7	2·0	6·7	1·7
	Mean	4·9	2·2	7·1	1·0

The specific gravities of roach, dace and carp skeletons and scales were determined, using a weighing bottle as a density bottle. The weight of the weighing bottle on successive fillings with water was found to be constant within a few milligrams (standard deviation for 10 trials = 7 mg.); this constancy was adequate for the determinations, in each of which about 2 g. of material was used. A vacuum pump was useful in removing bubbles of air from the material. The results are given in Table 4.

Table 4. *The specific gravity of bone*

	S.G. of skeleton	S.G. of scales
Roach	1·85	1·91
Dace	2·04	1·95
Carp	1·57, 1·67	1·56

Although there are clear interspecific differences in the specific gravity of bone, there is no appreciable difference between the bone of the skeleton and that of the scales within the species. In the following discussion the specific gravity of roach bone will be taken as 1·9, of dace bone as 2·0 and of carp bone as 1·6.

The specific gravity of fish tissues other than bone, scales, fat and swimbladder gas will be taken to be 1·06, a mean value which anticipates the results of some calculations to be presented in Table 5. This value is used for the following calculation only.

The volume of 1 g. of fat of specific gravity 0·91 is 1/0·91 ml. The volume of 1 g of tissue of specific gravity 1·06 is 1/1·06 ml. The replacement of 1 g. of such tissue by 1 g. of fat in a fish will increase the volume of the fish by (1/0·91) − (1/1·06) = 0·16 ml. This is equivalent to increasing the volume of the swimbladder by

0·16 ml. The acquisition by a fish of fat constituting 1% of its body weight will reduce the percentage volume of swimbladder required for neutral buoyancy by only 0·16. The variations in fat content found in Cyprinidae can thus have little influence on the volume of the swimbladder.

The volume of 1 g. of bone of specific gravity 1·8 (the mean of the specific gravities found) is 1/1·8 ml. The acquisition by a fish of bone constituting 1% of its body weight will increase the percentage volume of swimbladder required for neutral buoyancy by (1/1·06) − (1/1·8) = 0·39. The variations of total bone content found in Cyprinidae must be important in determining the volume of the swimbladder.

Table 5. *Summary of data on the sinking factors of roach, dace and carp*

Species (*a*)	Tissue (*b*)	Weight (% body wt.) (*c*)	Volume (ml./100 g. body wt.) (*d*)	Specific gravity (*e*)	Weight in water (c − *d*) (% body wt.) (*f*)
Roach	Bone	**8·1**	4·3	**1·9**	+3·8
	Swimbladder	0	**9·9**	0	−9·9
	Rest	91·9	85·5	1·07	+6·4
	Total	100	99·7	**1·003**	+0·3
Dace	Bone	**5·5**	2·8	**2·0**	+2·7
	Swimbladder	0	**8·1**	0	−8·1
	Rest	94·5	89·0	1·06	+5·5
	Total	100	99·9	**1·001**	+0·1
Carp	Bone	**7·1**	4·4	**1·6**	+2·7
	Swimbladder	0	**5·8**	0	−5·8
	Rest	92·9	89·6	1·04	+3·3
	Total	100	99·8	**1·002**	+0·2

The above discussion has shown that, in discussing the sinking factors of Cyprinidae, bone must be considered but fat is much less important. In this discussion a value of 1·06 was assumed for the specific gravity of tissues other than swimbladder, bone and fat. In Table 5 the actual values of this specific gravity of 'the rest' in roach, dace and carp are calculated from the data obtained on bone, swimbladders and sinking factors. The figures in bold type are data from this and a previous paper (Alexander, 1959); the remainder is calculated from them or is self-evident.

From column (*f*) of the table we find that the difference in bone content accounts for (3·8 − 2·7) = 1·1 of the difference in percentage volume of the swimbladder (1·8) between roach and dace. The remainder of the percentage volume difference is due to a small difference in the specific gravity of 'the rest'. The difference in percentage volume between dace and carp is entirely due to a difference in the specific gravity of 'the rest'.

The specific gravity of 'the rest' is calculated to be 1·07 in the roach, 1·06 in the dace and 1·04 in the carp. It was thought possible that differences in the specific gravity of muscle might contribute to these differences.

Muscle

The specific gravity of the muscle of roach and carp was determined by means of Hammerschlag's (1890) method for the specific gravity of blood, as used by Chen (1931) for the various tissues of the goldfish.

A small piece of muscle dissected from the tail of the fish was placed in a mixture of chloroform and benzene. By adding one or other of these substances the specific gravity of the mixture was adjusted until, as far as possible, the muscle neither floated nor sank. It was then determined by means of a hydrometer. The mixture was thoroughly stirred after each addition of fluid by means of a perforated plate stirrer, and care was taken to see that no bubbles of air clung to the muscle.

The results are given in Table 6. The mean specific gravity of roach muscle is found to be 1·063 and of carp muscle 1·046. It is hard to understand how this comes to be, as the fat contents of both are low, and they contain similar proportions of protein (Jacquot & Creac'h, 1950; the edible parts of roach contain 19·5 % protein, of carp 17·5–18·9 %). This helps, however, to explain the difference found in the specific gravity of the parts other than bone and swimbladder of the two species.

Table 6. *The specific gravity of muscle*

Species	Weight (g.)	S.G. of tail muscle	
Roach	23	1·062, 1·062	Mean 1·062
	23	1·064	1·064
	Mean		1·063
Carp	45	1·048, 1·046	1·047
	42	1·046, 1·041, 1·045	1·044
	Mean		1·046

SUMMARY

1. The mean sinking factor of each of eight species of Cypriniformes has been determined by a method involving measurement of the pressure at which anaesthetized specimens float. It was found to lie between 1001 and 1004 for all species except the gudgeon, for which it is 1008.

2. Roach, dace and carp have swimbladders of very different sizes. It was shown that differences in the weight and density of the skeleton and scales, and in muscle density, are important factors in determining the volume of swimbladder gas necessary for neutral buoyancy. Differences in fat content are unimportant in these fish.

I wish to thank Dr K. E. Machin and Dr G. M. Hughes for much advice, and the Development Commission for financial support.

REFERENCES

ALEXANDER, R. McN. (1959). The physical properties of the swimbladder in intact Cypriniformes. *J. Exp. Biol.* **36**, 315–32.

CHEN, S. C. (1931). The specific gravity of various body-parts, organs and tissues of wild and domesticated goldfish, *Carassius auratus*. *Biol. gen.* **7**, 555–74.

HAMMERSCHLAG, A. (1890). Über eine neue Methode zur Bestimmung des spezifischen Gewichtes des Blutes. *Wien. klin. Wschr.* **3**, 1018. Referred to by Chen (1931).

JACQUOT, R. & CREAC'H, P. V. (1950). Les protides du poisson et leur valeur alimentaire. *Notes et rapports de l'Office scientifique et technique des Pêches maritimes.* (N.S.), **6**, pp. 48.

JENKINS, J. T. (1925). *The Fishes of the British Isles.* London.

JONES, F. R. H. & MARSHALL, N. B. (1953). The structure and functions of the teleost swimbladder. *Biol. Rev.* **28**, 16–83.

LOWNDES, A. G. (1942). The displacement method of weighing living aquatic organisms. *J. Mar. Biol. Ass. U.K.* **25**, 555–74.

MOEHRES, F. P. (1940). Untersuchungen über die Frage der Wahrnehmung von Druckunterschieden des Mediums (Versuche an Bodenfischen). *Z. vergl. Physiol.* **28**, 1–42.

PLATTNER, W.-A. (1941). Études sur la fonction hydrostatique de la vessie natatoire des poissons. *Rev. suisse Zool.* **48**, 201–338.

POPTA, C. M. L. (1910). Étude sur la vessie aérienne des poissons. *Ann. Sci. nat. Zool.* (ser. 9), **12**, 1–160.

SPECTOR, W. S. (ed.) (1956). *Handbook of Biological Data.* Philadelphia, London.

THE PHYSICAL PROPERTIES OF THE ISOLATED SWIMBLADDER IN CYPRINIDAE

By R. McN. ALEXANDER

*Department of Zoology, University of Cambridge**

(*Received* 22 *October* 1958)

INTRODUCTION

The experiments described in previous papers (Alexander 1959*a*, *b*) were done on intact fish. They provide information only about the swimbladder as a whole. This paper will report experiments on isolated swimbladders designed to provide information on the parts of the swimbladder.

Müller (1843) reports that the anterior sac of the swimbladder is more extensible than the posterior, but gives no quantitative data. The method described below has been used to determine the extensibility and size of each of the sacs of the swimbladder. A number of species was examined in an attempt to elucidate the difference in the overall extensibility of the swimbladder reported in Alexander (1959*a*).

METHOD

The apparatus is shown in Fig. 1. The hypodermic needle was of size 20 and was bound into a piece of rubber tubing with copper wire as shown in the inset. It was blunted by rubbing its tip on emery paper to reduce the danger of puncturing the swimbladder. The capacity of the capillary was 0·0270 ml./cm. length. This apparatus was suitable for investigating the swimbladders of Cyprinidae weighing 50–120 g.

The experimental procedure was as follows. The fish was killed by a blow on the head, dried with a towel, and weighed. The swimbladder was removed with part of the pneumatic duct, great care being taken to avoid tearing the tunica externa. The hypodermic needle was then passed up the pneumatic duct so that its tip was in the posterior sac, and the duct wall was bound to this cannula with cotton thread.

The flask was filled with Ringer solution (Young, 1933; formula recommended for freshwater teleosts other than eels), the swimbladder on its cannula was inserted, and the bung pushed home. Sometimes a little air remained in the flask, but this was unimportant as the pressure in the flask was constant throughout the experiment; only the pressure in the swimbladder was altered. Ringer was passed in or out through the funnel to move the meniscus to a convenient position in the capillary.

A series of readings of meniscus position was then taken with the pressure in the swimbladder alternately at 2 and 6 cm. Hg. As the meniscus did not come to rest until about 30 sec. after each change of pressure, readings were taken at 1 min.

---

* Now at the Department of Zoology, University College of North Wales, Bangor.

intervals, the pressure being changed immediately after each reading. The first two readings were usually discarded as they often showed a greater change than subsequent ones, which remained constant within reasonable limits for a series of cycles. The meniscus movements represented changes in the volume of the swimbladder.

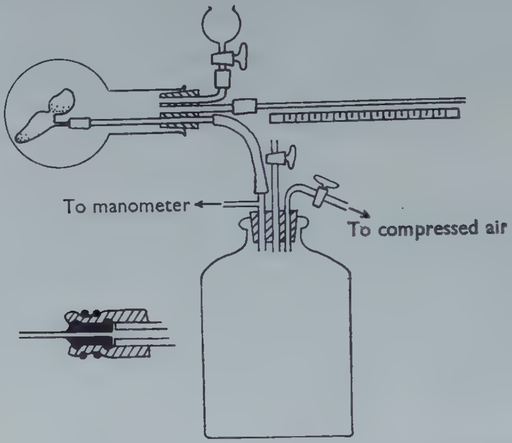


Fig. 1. The apparatus. The inset shows how the hypodermic needle which is passed up the pneumatic duct is bound with copper wire into a piece of rubber pressure tubing.

The pressure was then returned to 2 cm. Hg and the cannulated swimbladder was taken out of the flask. A cotton thread was tied tightly round the ductus communicans* to prevent the passage of gas between the two sacs. The swimbladder was returned to the flask and the readings were repeated. Whereas the first series of readings had given the extension of the whole swimbladder, the second, on account of the ligature, gave only that of the posterior sac.

The pressure was returned to 2 cm. Hg and the swimbladder removed from the flask. A half-hitch was tied round the pneumatic duct with a cotton thread, and the duct was slipped off the hypodermic needle while the knot was pulled tight. One or two more knots made the ligature secure.

The isolated swimbladder now had ligatures on the ductus pneumaticus and communicans, and an excess pressure of 2 cm. Hg in each sac. It remained to determine approximately the volume of each sac. This was done using a weighing bottle as a density bottle.

The whole swimbladder was weighed in Ringer in the bottle. It was necessary to insert the stopper slowly and wait some minutes for equilibration on account of the tendency to compress the swimbladder. The ductus communicans was then cut anterior to the ligature and the posterior sac alone weighed with Ringer in the bottle. The bottle was then weighed full of Ringer alone. From these three weighings, and the specific gravity of Ringer, it was possible to calculate the volume of each sac of the swimbladder. The weight of the swimbladder wall in Ringer was found to be negligible.

---

* This term is used by Evans (1925) for the connexion between the two sacs.

From these measurements of volume and volume change the extensibility of each sac of the swimbladder over the range 2–6 cm. Hg was calculated. The lower pressure, 2 cm. Hg, was selected so that the results would be readily comparable with the data on extension given in Alexander (1959*a*). The higher pressure was chosen to give conveniently large volume changes.

The use of the isolated swimbladder makes this experiment a very rough one. It was designed only to detect gross differences between the two sacs and between species.

RESULTS

There are two quantities which can be calculated either from the results of the experiments described here or from the results of those described in Alexander (1959*a*). These are the extensibility of the whole swimbladder over the range 2–6 cm. Hg excess pressure, and the volume of the swimbladder with 2 cm. Hg excess pressure. These data, as obtained from the two sets of experiments, are compared in Table 1.

Table 1. *Comparison of data obtained from intact fish and isolated swimbladders*

	Mean extensibility (%/cm. Hg between 2 and 6 cm. Hg)		Mean vol. at 2 cm. Hg excess pressure (ml. as % of body weight in g.)	
	Intact fish	Isolated swimbladder	Intact fish	Isolated swimbladder
Roach	1·5	2·3	9·3	8·1
Rudd	1·6	1·8	9·0	—
Dace	2·6	2·1	8·4	8·2
Tench	1·0	1·5	6·8	7·4
Bream	1·0	1·4	7·2	6·3
Carp	3·1	2·3	5·5	7·7
Crucian carp	1·0	1·0	8·7	8·9

Agreement between the two methods is very rough. Differences may be attributed to the crude nature of the experiments on isolated swimbladders and to the small number of specimens used. Very considerable stretching of carp swimbladders occurred in these experiments, leading to results very different from those for intact fish.

The experiments on isolated swimbladders were designed to determine the extensibility and size of each of the sacs of the swimbladder. The mean values of these for each species are shown in Table 2; details of individual fish are given in the Appendix. Four swimbladders whose tunicae externae were torn, and one from which it was missing, are included in the means; the extensibilities of the anterior sacs of these are not appreciably different from those of intact anterior sacs of the same species.

From these data a number of conclusions may be drawn. Müller's report of the difference in extensibility between the sacs is amply confirmed: in most species the anterior sac is about ten times as extensible as the posterior. As the anterior sac alone is involved in the operation of the Weberian ossicles, it is clearly advantageous to limit the extension of the swimbladder to this sac.

The more extensible anterior sac is usually rather smaller than the posterior sac, but in the carp it is very much larger than the posterior sac. The high overall extensibility of the carp swimbladder is due to this peculiarity, while that of the dace appears to be due to the unusual extensibility of a rather small anterior sac. The extensibility of the bream swimbladder only approaches its low asymptotic value at high pressures, and the experiments on isolated swimbladders were conducted over too low a range of pressures to show this exceptional inextensibility.

Table 2. *Data obtained from isolated swimbladders*

	Vol. of anterior sac / Vol. of posterior sac	Extensibility (%/cm. Hg between 2 and 6 cm. Hg)	
		Anterior sac	Posterior sac
Roach	0·6	4·8	0·7
Rudd	0·5	4·2	0·6
Dace	0·4	6·6	0·5
Tench	0·7	3·0	0·2
Bream	0·7	2·6	0·5
Carp	5·2*	2·8	0·1
Crucian carp	0·8	2·0	0·3

* Mean value for three unstretched specimens at normal excess internal pressure: 8·8.

DIFFERENTIAL EXTENSIBILITY

Guyénot (1909) tied a cannula into the posterior sac of an isolated tench swimbladder and inflated it. He photographed it at two unspecified excess pressures. He measured his photographs, and reported that the anterior sac was considerably more extensible in length than in breadth. He interpreted this as an adaptation to efficient operation of the Weberian mechanism.

This experiment has been repeated, again using a tench. The tunica externa was not damaged. The excess pressure was increased slowly from zero to 18 cm. Hg over a period of 27 min., and a photograph was taken from a dorsal viewpoint after every cm. Hg increase. The camera was mounted at a fixed height above the swimbladder. The photographic lights were turned off between photographs to avoid drying the specimen.

The negatives were projected in an enlarger at constant magnification and their outlines drawn. In each outline the length of the anterior sac from the ductus communicans to the insertion of the ossicles and the width across the widest part were measured. Both length and width were found to increase linearly with pressure above 3 cm. Hg: below this pressure the increase was more rapid.

When the excess pressure was increased from 3 to 18 cm. Hg the length of the swimbladder increased from 4·80 to 5·47 units—an increase of 14%. For the same increase in pressure the width of the swimbladder increased from 2·93 to 3·32 units—an increase of 13%. There is thus no appreciable difference between the extensibilities in these two directions.

A similar but less numerous set of photographs was taken from a lateral viewpoint. No marked difference between the extensibilities of the anterior sac in length and in depth was found.

My failure to find greater extensibility in length than in breadth in the tench anterior sac is difficult to reconcile with Guyénot's result. His diagram illustrating his measurements, however, provides a clue (Guyénot, 1909, fig. 4). It shows his two measurements of breadth as having been taken at the same absolute distance from the ductus communicans. They should rather have been taken between the same points on the surface of the swimbladder, which extends in length as well as in breadth. My measurements were made at a fixed level on the swimbladder—its broadest, parallel-sided part, about one-quarter of the anterior sac length from its anterior end.

Guyénots diagram is reproduced, with some additions, as Fig. 2. His measurements of breadth were made at *bb′*, which is 0·24 of the anterior sac length from its posterior end at the lower pressure, but only 0·21 at the higher. The sharp slope of the walls here makes this misplacement of measurements important. I would have measured the swimbladder at *ee′* (lower pressure) and *ff′* (higher presure).

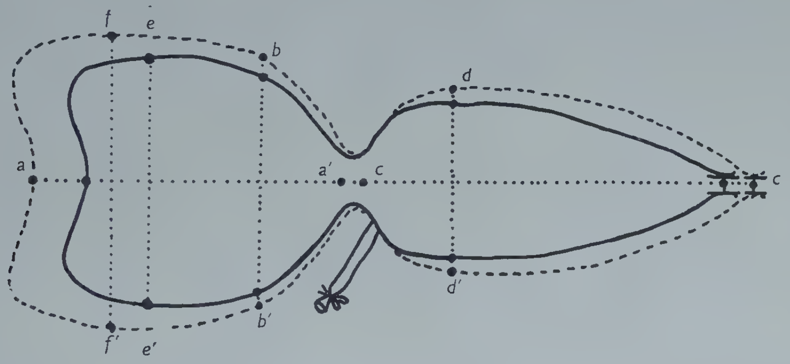


Fig. 2. Reproduced from Guyénot (1909) with additions. Explanation in the text.

The proportions of Guyénot's figure agree so well with his published measurements that we may conclude that the figure was traced from the photographs. *ff′* is 19% longer than *ee′*. This increase is not much less than the 24% increase in *aa′*, and is very much larger than the 9% increase in *bb′*.

It thus appears that proper treatment of Guyénot's data leads to a conclusion similar to mine: that there is no marked difference between the extensibilities of the anterior sac in length and in breadth.

Thus one reported adaptation of the ossicle system is confirmed and another denied; it is found that the anterior sac is much more extensible than the posterior, but that it is not significantly more extensible in length than in other directions.

SUMMARY

1. The anterior sac of the cyprinid swimbladder is much more extensible than the posterior sac.

2. The anterior sac is not significantly more extensible in length than in other directions.

I wish to thank Dr K. E. Machin and Dr G. M. Hughes for much advice, and the Development Commission for financial support.

REFERENCES

Alexander, R. McN. (1959*a*). The physical properties of the swimbladder in intact Cypriniformes. *J. Exp. Biol.* **36**, 315–32.

Alexander, R. McN. (1959*b*). The densities of Cyprinidae. *J. Exp. Biol.* **36**, 333–40.

Evans, H. M. (1925). A contribution to the anatomy and physiology of the air-bladder and Weberian ossicles in Cyprinidae. *Proc. Roy. Soc.* B, **97**, 545–76.

Guyénot, E. (1909). Les fonctions de la vessie natatoire des poissons téléostéens. *Bull. sci. Fr. Belg.*, ser. **7**, t. 43, 203–96.

Müller, J. (1843). Untersuchungen über die Eingeweide der Fische, Schluss der vergleichenden Anatomie der Myxinoiden. *Abh. K. Akad. Wiss., Berl.*, pp. 109–70.

Young, J. Z. (1933). The preparation of isotonic solutions for use in experiments with fish. *Pubbl. Staz. zool. Napoli*, **12**, 425–31.

APPENDIX

Details of results

Species	Specimen	Vol. of anterior sac / Vol. of posterior sac	Extensibility in %/cm. Hg, between 2 and 6 cm. Hg	
			Anterior sac	Posterior sac
Roach	P 3*	0·5	5·2	0·5
	P 25	0·7	4·4	0·8
	Mean	0·6	4·8	0·7
Rudd	P 2	0·5	4·2	0·6
Dace	P 31	0·3	6·4	0·4
	P 32	0·4	6·7	0·5
	Mean	0·4	6·6	0·5
Tench	P 5	—	2·3	0
	P 6	0·6	4·1	0·1
	P 8	0·5	—	—
	P 9	1·1	2·9	0·4
	P 10	0·7	2·5	0·3
	Mean	0·7	3·0	0·2
Bream	P 22	0·6	2·4	0·4
	P 23	0·8	1·6	0·7
	P 24	0·7	3·7	0·5
	Mean	0·7	2·6	0·5
Carp	P 11	3·6	3·0	0
	P 12	7·5	3·1	0
	P 13*	4·5	2·3	0·4
	Mean	5·2	2·8	0·1
Crucian carp	P 4	—	—	0·3
	P 16*	0·6	2·0	0
	P 17	—	—	0·3
	P 18	0·7	2·7	0·3
	P 19*	0·8	1·5	0·3
	P 21*	0·9	1·9	0·3
	Mean	0·8	2·0	0·3

* Indicates that the tunica externa was torn or missing.

THE PHYSICAL PROPERTIES OF THE SWIMBLADDERS OF FISH OTHER THAN CYPRINIFORMES

By R. McN. ALEXANDER
*Department of Zoology, University of Cambridge**

(*Received* 22 *October* 1958)

INTRODUCTION

The experiments and observations reported in previous papers (Alexander, 1959*a*–*c*) have all been made on Cypriniformes. The swimbladders of these fish are specialized for the operation of the Weberian ossicles. An excess internal pressure is probably essential to the functioning of the ossicles. Outside the Cypriniformes the excess internal pressure has been measured in a 'Vieille de mer' (probably *Labrus bergylta*) by Chabry (1884) who found a pressure of 5–10 cm. of water, and in perch by Evans & Damant (1928) who could detect no excess internal pressure. Chabry does not describe any precautions to ensure that the fish was adapted to the depth at which it was tested. It seemed desirable to make a survey to determine whether the possession of an excess internal pressure is widespread or a peculiarity of the Cypriniformes.

In the work described in a previous paper (Alexander, 1959*a*) the changes in swimbladder volume, which occur as the external pressure changes from the value to which the fish is adapted, were investigated for Cypriniformes. They were found to be smaller, and sometimes very much smaller, than would occur in an unconstrained bubble of gas of the same initial volume. Where there is an excess internal pressure the relative sensitivity must be less than 1; the volume changes will be less than for a free bubble when the pressure is increased as well as when it is decreased. Where there is no excess internal pressure, unless either the swimbladder wall or the body wall is rigid in all its parts, they cannot limit volume changes as the external pressure is increased. They could, if they became taut as the pressure fell, limit the volume changes consequent upon a decrease of pressure. Jones (1951) found not only that the relative sensitivity of the perch swimbladder is 1, but also that when the pressure on a perch adapted to life in a shallow aquarium was reduced by 30 cm. Hg, the volume change was 80% of that to be expected of a free bubble. The swimbladder wall of the perch can thus be of no importance in limiting its change of buoyancy with depth. It seemed important to determine if this condition was the normal one, and that of the Cypriniformes a striking departure from it.

The following experiments were carried out to investigate these problems. The volume of the swimbladder and the sinking factor were also determined for each species examined.

---

* Now at the Department of Zoology, University College of North Wales, Bangor.

The marine species were studied at the Plymouth laboratory of the Marine Biological Association. It was not possible to control the depths of the aquaria, and tanks of various depths up to 1 m. were used. Nor was it always possible to allow the desirable 24 hr. for acclimatization. It was usually possible in such cases to determine whether the fish had adjusted its buoyancy to suit the depth of the tank by comparing its behaviour with that of healthy specimens of the same species on permanent exhibition in the aquarium.

EXPERIMENTS

Excess internal pressure

The majority of fish which possess swimbladders but are not Cypriniformes are physoclists. Gas cannot therefore be withdrawn from their swimbladders by reducing the pressure, and the technique described in a previous paper (Alexander, 1959*a*) cannot be applied to them as it stands. The following method was used in an attempt to detect excess internal pressure. A direct manometric method was discarded after trial as unsuccessful.

Part I of the procedure described in Alexander (1959*a*) was carried out. Readings were taken at 0, +6 and +18 cm. Hg. It was not necessary to use water of reduced air content.

Boyle's Law was used to calculate the volume of the swimbladder from the volume change between 0 and +6 cm. Hg, assuming that the pressure of the swimbladder gases was equal to the external pressure throughout. The same was done from the volume change between 0 and +18 cm. Hg. If, as assumed, there was no excess internal pressure, the two results should be identical. If there was an excess internal pressure, the former result should be the smaller.

The sensitivity of this method of detecting excess internal pressure is limited by the accuracy with which the smaller volume changes can be measured. The differences between the volumes calculated from the 6 cm. Hg readings and those calculated from the 18 cm. Hg. ones for thirty-two individual fish outside the Cypriniformes all lay between +6% and −6%, with standard deviation 3·6% and mean −0·31%, which is not significantly different from zero (*t* test, $P \simeq 0{\cdot}6$). The differences are thus probably due to experimental error, and there is no evidence of widespread excess internal pressure outside the Cypriniformes. It remains possible that a few of the fish may have had an excess internal pressure.

In the case of the pike, a physostome which releases gas readily at reduced pressures, it was possible to carry out the full routine (Alexander, 1959*a*). Three pike which gave differences of −3%, −4% and −6% in the test here described were found to have excess internal pressures of −0·4, +0·3 and +0·3 cm. Hg: that is to say, any excess internal pressure they may have had was too small to be measured by the method. The only fish outside the Cypriniformes which gave a negative difference as large as −6% were the pike mentioned above and an eel. There is therefore no case for ascribing an excess internal pressure to any individual among these fish.

The sensitivity of the test can be gauged by applying it to Cypriniformes of known excess internal pressure. This was done, using the data previously collected (Alexander, 1959*a*) for those specimens which had excess internal pressures less than 3 cm. Hg. The results suggested that a fish having an excess internal pressure greater than 2·0 cm. Hg. would be most unlikely to show a difference within the range, +6% to −6%, found for fish outside the Cypriniformes. Only one minnow with an excess internal pressure of 2·2 cm. Hg and a difference of −5% breaks this rule.

It must be emphasized that no attempt is being made to establish an exact relationship between excess internal pressure and the result of the test. Extensibility would have to be considered in such an attempt. But it seems possible to conclude that the mean excess internal pressure of the thirty-two fish examined from orders other than the Cypriniformes was very close to zero, and that it is unlikely that any one of these fish had an excess internal pressure greater than 2·0 cm. Hg. The results of the test are given in detail in the Appendix.

None of the fish examined had rigid swimbladder or body walls. Zero excess internal pressure would therefore imply a relative sensitivity of 1.

Percentage volume and sinking factor

As these fish had no detectable excess internal pressure, the volumes calculated for the swimbladder gas from the volume change as the external pressure was increased by 18 cm. Hg should be the true volumes of the swimbladders. Since the fish were weighed, the percentage volumes of their swimbladders could be calculated. They are given in the Appendix and summarized in Table 1.

The available specimens of *Gobius flavescens* were too small for satisfactory determination of the swimbladder volume by this means. The following method was adopted, as it seemed interesting to know the size of the swimbladder in this, the only pelagic species among the British gobies; the other gobies have reduced swimbladders. The flotation pressures of three specimens were determined and the specimens were weighed. They were then placed in a wide-mouthed density bottle which was filled with distilled water and weighed. They were cut in half transversely at the level of the swimbladder, whose gases were allowed to escape, and the weighing in the density bottle was repeated. Care was taken to avoid introducing bubbles of air, e.g. in their mouths, into the density bottle. The difference in grammes between the two weighings equalled the total volume of the swimbladder gases in millilitres. It was not possible to test this species for excess internal pressure.

The sinking factors of the species discussed here were determined in the manner described elsewhere (Alexander, 1959*b*). The flotation pressures of marine species were, of course, determined in sea water. The relative sensitivity was in all cases taken to be 1. Where 'dense' is given for the sinking factor, the fish failed to float at −24 cm. Hg and the sinking factor could not be determined by this method. The specimens of *Esox*, *Anguilla* and *Conger* were too long for it to be possible to determine their flotation pressures in my apparatus.

Table 1. *Mean percentage volume of the swimbladder, and mean sinking factor, for fish other than Cypriniformes*

Species	Mean % vol.	Previous values	Mean S.F.	Previous values
		Freshwater species		
Salmo trutta	5·1	—	1008	—
Esox lucius	4·9	—	—	—
Anguilla anguilla	3·7	—	—	—
Anguilla anguilla (silver eel stage)	1·3	—	—	—
Perca fluviatilis	—	7·94 (Plattner, 1941)	1001	1010* (Plattner, 1941)
	—	7·5† (Jones, 1951)	—	1005 (Jones, 1951)
Acerina cernua	8·7	—	1000	—
		Marine species		
Conger conger	3·5	4·7 (Plattner, 1941)	—	993 (Plattner, 1941); 1000 (Lowndes, 1942)
Gadus luscus	4·9	5·0 (Plattner, 1941)	998	1001 (Plattner, 1941)
G. merlangus	3·6	—	999	—
Onos mustelus	1·5	0·7 (Plattner, 1941); 1·1 (Jones, 1951)	Dense	1060 (Plattner, 1941); 1024 (Jones, 1951)
Spinachia spinachia	5·5	—	1004	—
Trachurus trachurus	4·2	—	1004	—
Labrus bergylta	5·5	—	1001	—
Crenilabrus melops	5·5	4·9 (Plattner, 1941); 4·9 (Jones, 1951)	1002	1004 (Plattner, 1941); 1005 (Lowndes, 1942); 1007 (Jones, 1951)
Gobius niger	2·2	—	Dense	—
G. paganellus	1·2	0·3 (Plattner, 1941)	Dense	1080 (Plattner, 1941)
G. minutus	2·0	—	Dense	—
G. flavescens	5·0	—	1002	—
Trigla gurnardus	5·3	—	1010	—

* Plattner's wrongly calculated mean is here corrected.

† This value given by Jones (1951) appears to be that reported by him in a thesis (Jones, 1950) as the mean for twenty-nine male perch. The thesis gives a mean of 6·2 for twenty-one female perch. All Jones's perch were mature fish caught in the spawning season.

The sinking factors found are shown with the swimbladder volumes in Table 1 and in more detail in the Appendix.

In many cases the values are based on one or two specimens only. The main object of these experiments was the search for excess internal pressure, and the accumulation of values for percentage volume and sinking factor was not thought sufficiently important to justify a more prolonged study.

Two of the values of percentage volume differ markedly from those of previous authors: those for *Conger* and *Gobius paganellus*. In the latter species the swimbladder is reduced, there is no question of buoyancy adjustment, and no great constancy of volume can be expected (cf. the differences between Plattner's, Jones's and my mean values for *Onos*, and the wide scatter, 0·3 –2·6, of my individual values for *Onos*). Plattner had only one and I had only two specimens of *G. paganellus*. In *Conger* the swimbladder is well developed, and my specimens appeared to be very close in density to sea water. But Plattner's specimen was much less dense than sea water, and his experiments on others whose swimbladder gases had been partly removed showed (see his graph, figure 5) that a density equal to sea water could be expected when the percentage volume was about 3·5.

The silver eel was denser than fresh water but considerably less dense than sea water. Its swimbladder was small, as were those of the bottom-living species (*Onos mustelus*, and the gobies other than *Gobius flavescens*). Many marine bottom-living teleosts have no swimbladder (e.g. *Blennius*, *Callionymus* and the flatfish). The remaining marine species (including *G. flavescens*) are pelagic in habit and were found to have sinking factors close to 1000 (the one specimen of *Trigla* was in poor condition) and mean percentage volumes between 3·5 and 5·5. The buoyant fresh-water species require rather larger swimbladders on account of the lower density of their medium; mean percentage volumes for them vary from 4·9 to 8·7. The corresponding range for the Cypriniformes (Alexander, 1959*a*) is 5·8–9·9.

Experiments at reduced pressures

The experiments described above demonstrate that the species examined have no excess internal pressure, and hence that their swimbladder walls can be of no importance in controlling swimbladder volumes at depths greater than that to which they are adapted. It remains possible that they might become important as the gases expand at reduced depths. A number of determinations were therefore made of swimbladder volume changes at reduced pressures. These were compared with the changes predicted by Boyle's Law from the volume changes occurring between 0 and +18 cm. Hg external pressure. When the pressure was restored and a reduction repeated a slightly larger volume change usually occurred, but close constancy was achieved in successive cycles. Apparently the elastic behaviour of the swimbladder wall is, as in Cypriniformes, slow. The steady volume change achieved in pressure cycles between −24 cm. Hg and manometer zero was recorded.

Negative buoyancy at reduced depths can normally only be a problem to fish whose sinking factors are near 1000. Five such fish were examined. The results for the pressure change 0 to −24 cm. Hg are shown in Table 2.

Table 2. *Volume changes between* 0 *and* −24 *cm. Hg*

Specimen	Observed meniscus displacement (cm.)	Predicted meniscus displacement (cm.)	Difference (%)
q 78. *Gadus merlangus*	21·9	21·0	+4
qq 23. *Spinachia spinachia*	33·6	36·3	−7
q 86. *Acerina cernua*	52·0	54·4	−5
qq 29. *Trachurus trachurus*	44·2	43·2	+2
qq 24. *Crenilabrus melops*	37·1	41·9	−11

The positive differences are clearly due to experimental error, but the volume changes of *Spinachia* and *Crenilabrus* were significantly less than the Boyle's Law prediction. The differences are, however, small—smaller even than Jones's (1951) mean difference of −14% for the perch (reduction of 25 cm. Hg).

Jones (1952) found that perch adapted to a given pressure could not maintain their stations without discomfort when the pressure was reduced by more than one-sixth, and not at all when the reduction was more than one-third. For perch adapted to 80 cm. Hg the critical reductions are 10–15 cm. Hg and about 25 cm. Hg. Marine fish, with rather smaller swimbladders, would require less energy to counteract negative buoyancy at a given pressure reduction, and may be expected to be able to withstand rather larger reductions.

My investigations, down to −24 cm. Hg, may be taken as covering at least the range of comfortable swimming of the species examined. Within this range any influence the swimbladder wall has on swimbladder volume is very slight. It is concluded that in *Gadus*, *Spinachia*, *Acerina* (a fresh water species), *Trachurus* and *Crenilabrus* the swimbladder wall has no importance in restricting buoyancy changes. Jones has found the same to be true of the perch. It seems probable that this condition is general outside the Cypriniformes. It is very markedly in contrast with the situation within the Cypriniformes. In Fig. 1 the volume change with pressure of a roach swimbladder is compared with that of *Spinachia.*

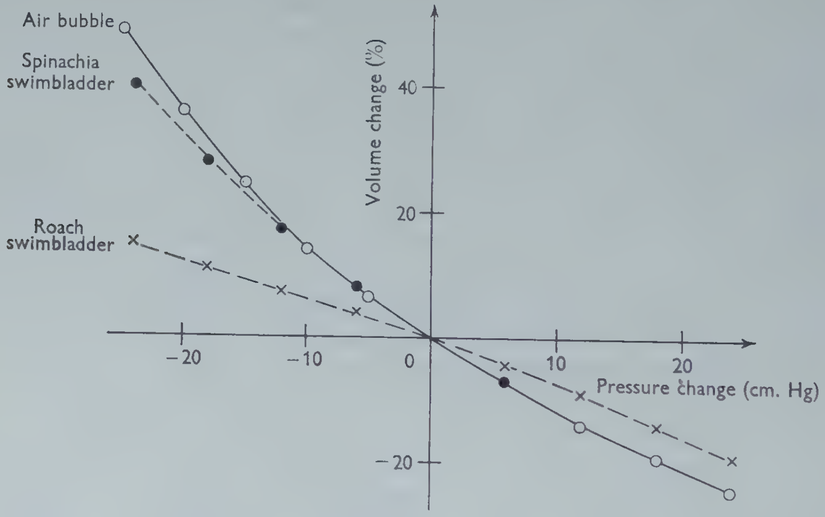


Fig. 1. Comparison of the changes of volume with external pressure of an air-bubble and of the swimbladders of *Spinachia* and roach adapted to life in shallow aquaria.

In a previous paper (Alexander, 1959*b*) the significance of the product of percentage volume and relative sensitivity was discussed. Outside the Cypriniformes the relative sensitivity is 1 and the product is equal to the percentage volume. The product is an index of the rate of change of buoyancy with pressure. Its importance is confined to species which have sinking factors close to 1000. In buoyant marine species it lies between 3·5 and 5·5—limits very similar to those found in Cypriniformes. In buoyant freshwater fish outside the Cypriniformes it lies between about

5 and 9. The lower value in marine fish is due to the adequacy of smaller swimbladders in the sea than in fresh water; that of the Cypriniformes, to a reduction in relative sensitivity.

Experiments on isolated swimbladders

It has been shown that the swimbladder wall has no importance in controlling swimbladder volume outside the Cypriniformes, at least in the species investigated. It is usually much more delicate than is normal in the Cypriniformes. It was felt that it would be interesting to investigate its elastic properties.

The method described (Alexander, 1959*a*) for determining extensibility in Cypriniformes cannot readily be adapted to other fish, in whose swimbladders only a slight excess pressure develops even at much reduced external pressures. It is, however, possible to calculate from Jones's (1951) results with the perch that its extensibility is uniformly 7·6%/cm. Hg over the range 1–3 cm. Hg. There is no sign of the change of extensibility with pressure reported for Cypriniformes.

The method previously described (Alexander, 1959*c*) was used to study the volumetric extension of trout and pike swimbladders. A ball-ended hypodermic needle was used to avoid unnecessary risk of puncture. The tunica externa of the pike is so firmly fixed to the vertebrae that it cannot be separated from them without damage. The vertebral column and a little of the muscle on either side of it was therefore left attached to the excised pike swimbladders.

The swimbladders of both species usually burst when the excess pressure in them exceeded 3 cm. Hg, whereas those of tench will stand pressures of 20 cm. Hg and perhaps much more. The very few readings which could be taken on the two trout in which the experiment was successful indicate an extensibility of 8–10%/cm. Hg between 1 and 3 cm. Hg, and do not suggest any change of extensibility with pressure. In pike, on the other hand, very marked plastic stretching occurs; in each of the two specimens examined the capacity of the swimbladder was about doubled when they were stretched by an excess pressure of 2 cm. Hg.

The swimbladders of pike and trout clearly have very different properties from those of the Cypriniformes.

SUMMARY

1. No excess internal pressure, such as has been found in the Cypriniformes, could be detected in the swimbladders of seventeen species from other orders. The swimbladder wall in these fish exercises no appreciable constraint on changes of buoyancy with depth.

2. Determinations of sinking factor and swimbladder volume are reported.

I wish to thank Dr G. M. Hughes and Dr K. E. Machin for much advice, the Marine Biological Association for the use of the facilities of the laboratory at Plymouth, and the Development Commission for financial support.

REFERENCES

ALEXANDER, R. McN. (1959*a*). The physical properties of the swimbladder in intact Cypriniformes. *J. Exp. Biol.* **36**, 315–32.

ALEXANDER, R. McN. (1959*b*). The densities of Cyprinidae. *J. Exp. Biol.* **36**, 333–40.

ALEXANDER, R. McN. (1959*c*). The physical properties of the isolated swimbladder in Cyprinidae. *J. Exp. Biol.* **36**, 341–46.

CHABRY, L. (1884). Sur l'équilibre des poissons. *J. Anat., Paris*, pp. 139–48.

EVANS, H. M. & DAMANT, G. C. C. (1928). Observations on the physiology of the swimbladder in cyprinoid fishes. *Brit. J. Exp. Biol.* **6**, 42–55.

JONES, F. R. H. (1950). The role of the swimbladder in regulating the vertical movements of teleostean fishes. A thesis presented for the degree of Ph.D. in the University of London.

JONES, F. R. H. (1951). The swimbladder and the vertical movements of teleostean fishes. I. Physical factors. *J. Exp. Biol.* **28**, 553–66.

JONES, F. R. H. (1952). The swimbladder and the vertical movements of teleostean fishes. II. The restriction to rapid and slow movements. *J. Exp. Biol.* **29**, 94–109.

LOWNDES, A. G. (1942). The displacement method of weighing living aquatic organisms. *J. Mar. Biol. Ass. U.K.* **25**, 555–74.

PLATTNER, W.-A. (1941). Études sur la fonction hydrostatique de la vessie natatoire des poissons. *Rev. suisse Zool.* **48**, 201–338.

APPENDIX

Details of results

Species	Specimen	Weight (g.)	% vol.	Sinking factor	Pressure test (%)
Salmo trutta	Q 21	65	4·6	1008	−1
	Q 23	60	5·5	—	−5
	Mean		5·1	1008	
Esox lucius	Q 35	140	4·7	—	−3
	Q 36	250	4·5	—	−4
	Q 37	160	5·6	—	−6
	Mean		4·9	—	
Anguilla anguilla	q 87	22	3·7	—	−6
Anguilla anguilla (silver eel stage)	q 74	95	1·3	—	+1
Conger conger	Q 101	165	3·8	—	−1
	Q 102	210	3·2	—	+2
	Mean		3·5		
Gadus luscus	Q 99	69	4·9	998	+3
G. merlangus	q 77	62	2·8	1006	+1
	q 78	23·5	4·4	992	+5
	Mean		3·6	999	
Onos mustelus	qq 26	1·8	2·6	Dense	+4
	qq 34	8·0	1·7	Dense	−5
	qq 79	30·5	0·3	Dense	—
	Mean		1·5		
Spinachia spinachia	qq 23	4·5	5·7	1006	−1
	qq 35	10·0	5·2	1001	+3
	Mean		5·5	1004	
Perca fluviatilus	Q 45	—	—	1001	−4
Acerina cernua	q 86	31	8·7	1000	0
Trachurus trachurus	qq 29	9·3	4·2	1005	+4
	q 76	45	4·2	1003	+4
	Mean		4·2	1004	
Labrus bergylta	qq 37	16·0	5·5	1001	−3
Crenilabrus melops	qq 22	3·6	6·0	997	+6
	qq 24	7·8	5·3	1001	+2
	qq 25	2·9	6·2	1005	0
	qq 36	6·6	5·3	1003	−1
	Q 103	66	4·5	1006	+3
	Mean		5·5	1002	
Gobius niger	qq 31	2·4	2·4	Dense	−5
	qq 38	14·2	2·1	Dense	+2
	qq 39	7·9	2·0	Dense	−1
	Mean		2·2		
G. paganellus	qq 30	14·3	0·8	Dense	−5
	qq 33	8·8	1·6	Dense	+4
	Mean		1·2		
G. minutus	qq 32	2·6	2·0	Dense	0
G. flavescens	3 fish	Total 2·03	5·0	1002	—
Trigla gurnardus	q 71	17·8	4·6	1010	−3

THE COMPOSITION OF THE BLOOD OF THE SHORE CRAB, *CARCINUS MOENAS* PENNANT, IN RELATION TO SEX AND BODY SIZE

II. BLOOD CHLORIDE AND SULPHATE

By A. B. GILBERT

Department of Zoology, University of Durham, King's College,
Newcastle upon Tyne

(*Received* 12 *November* 1958)

INTRODUCTION

In a previous communication it has been shown that in both sexes of the shore crab blood conductivity rises steadily to a maximum at a weight of approximately 35 g. and thereafter falls with increase in body weight: on the other hand total osmotic pressure (O.P.) shows a small but steady decrease with increasing size.

It seemed worthwhile therefore to consider the ionic composition of the blood in relation to sex and body size; such information as is available does not consider the factor of size and in only one case has the influence of sex been examined (Drihlon Courtois, 1934). In the present work total blood chloride and sulphate were investigated.

MATERIALS AND METHODS

Procedures were essentially similar to those already described (Gilbert, 1959), crabs again being obtained from the shore close to the Dove Marine Biological Station, Cullercoats.

Blood was removed through a small incision in the arthrodial membrane at the base of the large chela. Using a glass pipette with a rubber teat sufficient blood was removed from a single crab for either chloride or sulphate determinations. Anti-clotting agents were not used.

The chloride ion is of such importance that in view of the conductivity results (Gilbert, 1959) variations with size might be expected. Moreover, the large amounts of chloride present, and the accuracy of the method for its determination made it likely that any differences would be detectable. Chloride was determined on 1 ml. samples by the method of Robertson & Webb (1939): the sample is treated with $AgIO_3$, the displaced iodine dissolved in KI and titrated with thiosulphate. Titrations were carried out until three consecutive readings were obtained within 0·005 ml. of one another, and the correction of Sendroy (1939) applied to the mean of the results. Since the ionic content of the blood depends on the external medium, the chloride of the sea water in which the crabs were kept was determined each day. However, only slight fluctuations were found over the course of several weeks and during the latter part of the investigation it was determined weekly.

The silver iodate was prepared in the laboratory at first, but later it was obtained commercially (B.D.H.); identical results were obtained with both preparations.

Blood sulphate

There is no accurate method for the semi-micro determination of the sulphate content of biological fluids. The most satisfactory yet developed is that of Robertson & Webb (1939) for which an accuracy of 2% is claimed; this could not be achieved in the present work. With test solutions values were usually low, and in the region of 93% of the true value, with a variation of ±3%. It was found that if the sodium sulphate used in the titration was rapidly added to within 0·5 ml. of the expected end-point and the titration then continued as described by Robertson & Webb the variation was reduced to about ±1%; but results were still low. The investigation was restricted to possible differences between the sexes as it was felt that the method was not sufficiently accurate for the demonstration of variations in blood sulphate with body size.

Sufficient blood could be obtained from a single crab for two and usually three determinations. The mean of these was taken.

All glassware was thoroughly washed in hot water, rinsed repeatedly in distilled water, and dried with acetone. No contamination from the sulphate present in the cleansing fluids usually used for cleaning glassware could be detected.

RESULTS

Blood chloride

For convenience the results of the determinations were converted to millimolar chloride. These values have been plotted against body weight in Fig. 1 for sixty-seven male and in Fig. 2 for sixty-three female crabs. It is obvious that blood chloride is not constant over the whole size range, and that for both sexes, the pattern is very similar to that found for blood conductivity (Gilbert, 1959). From a value of about 490 mM. for the smallest animals blood chloride increases steadily to a maximum of about 520 mM. at a body weight of about 35 g. For crabs above this weight there is a gradual decrease until it reaches a value of about 500 mM. for the largest animals.

For the statistical analysis, the data for each sex was divided into two groups, above and below 35 g., as for the conductivity results (Gilbert, 1959); reference to Figs. 1 and 2 will show that this treatment is justified. The respective regression coefficients were calculated by the method of least squares. As would be expected, all four regression lines differ significantly from the horizontal ($P < 0{\cdot}01$ for females below 35 g. and $P < 0{\cdot}05$ for the others).

An analysis of co-variance showed that there was no significant difference between the two regression coefficients for the lines of positive slope for males and females below 35 g. body weight, nor between the two regression coefficients for lines of negative slope for animals above 35 g. ($P > 0{\cdot}05$ in each case). Throughout the size range females tended to have higher values of blood chloride than males. Below

35 g. body weight this difference is highly significant ($P < 0{\cdot}01$); but it was not significant for crabs above this weight ($P > 0{\cdot}05$).

In view of the conductivity results (Gilbert, 1959) this was rather surprising. Above 35 g. body weight the males had a higher conductivity, but there is no difference in the chloride content of the blood; below 35 g. body weight there was no difference in the conductivity yet the males have a lower chloride value than the females.

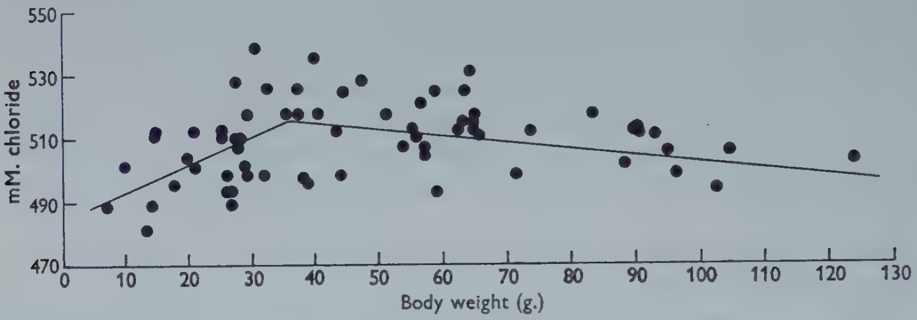


Fig. 1. Body weight and blood chloride in male crabs. Linear regression lines were calculated (1) for animals below 35 g. body weight, and (2) for animals above this weight. This procedure was followed for all subsequent figures except Fig. 3.

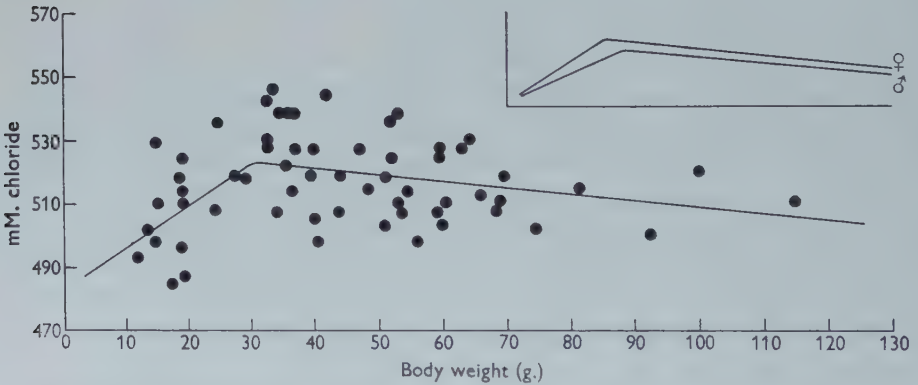


Fig. 2. Body weight and blood chloride in females. Inset: calculated regression lines for males and females.

Blood sulphate

The disparity between the results obtained for conductivity and blood chloride suggested that the concentration of some of the other ions differed between the sexes. Moreover, the chloride results suggested that such a difference would be among the anions. There is already some evidence that the blood phosphate may be higher in males than in females (Drihlon Courtois, 1934). In the present work blood sulphate was investigated in view of the greater concentration of this ion (Webb, 1940).

The results of the determinations have been converted to millimolar sulphate and plotted against body weight in Fig. 3 for thirty-one male and twenty-seven female crabs. As already mentioned, the method for the sulphate determination is not sufficiently accurate to enable differences with body size to be detected; clearly Fig. 3 shows no trend with body weight and a straightforward comparison of the two means is justified. The mean value for males is 15·1 and that for females over the same size range is 11·3. The difference of 3·8 between the two sexes is highly significant ($P < 0{\cdot}01$).

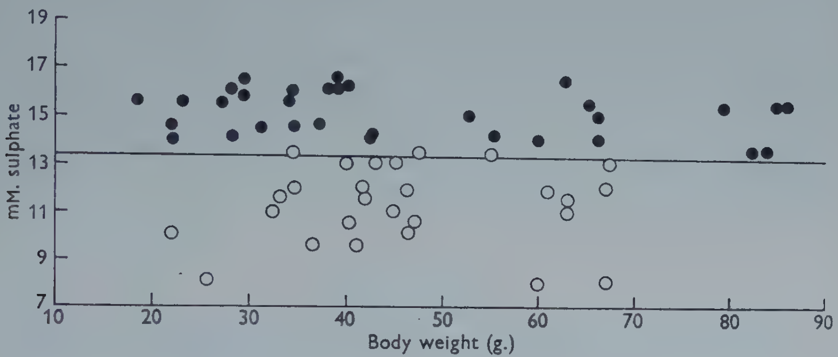


Fig. 3. Body weight and blood sulphate for male and female crabs. The horizontal line represents the population mean. ●, Males; ○, females.

DISCUSSION

Clearly failure to consider sex or body size when comparing the ionic composition of the blood of the shore crabs could be responsible for the lack of agreement between the results reported in the literature (Quinton, 1904; Duval, 1925; Bateman, 1933; Drihlon Courtois, 1934; Nagel, 1934; Webb, 1940; Shaw, 1955; and others). The sulphate content of the blood of female crabs has been shown to be significantly lower than that of males. Blood chloride varies with body size and sex, the pattern being very similar to that for the conductivity, in that the maximum value is reached at a weight of about 35 g. with a general decrease above and below this weight. In general the blood chloride of the males tends to be lower than that of the females; below 35 g. this difference is statistically significant, although it is not so above this weight. It has also been shown that there is no significant difference in conductivity of the blood between the males and females for weights above 35 g. Males tend to grow at a greater rate than females (Orton, 1936) so that at any given weight the males will be younger. If the sexes were compared by age rather than by body weight any differences would be accentuated if they were related to maturity, so that it is possible that they would be significant throughout.

A detailed investigation of the ionic composition of the blood has already been reported for animals also supplied from Cullercoats (Shaw, 1955). Crabs used by this worker were probably females of 35–40 g. body weight (Personal communication). On this estimate of sex and size Shaw's results and mine for both blood chloride and conductivity determinations show good agreement: his figure for chloride is 524 and that for conductivity 552; for females of 35 g. body weight my values are 523 and 556 respectively. Although it would appear therefore to be justified to use the results of Shaw for crabs of this size and sex, caution is necessary since a comparison of the data of Shaw (1955) and Webb (1940) suggests that crabs of different 'races' may have different blood compositions.

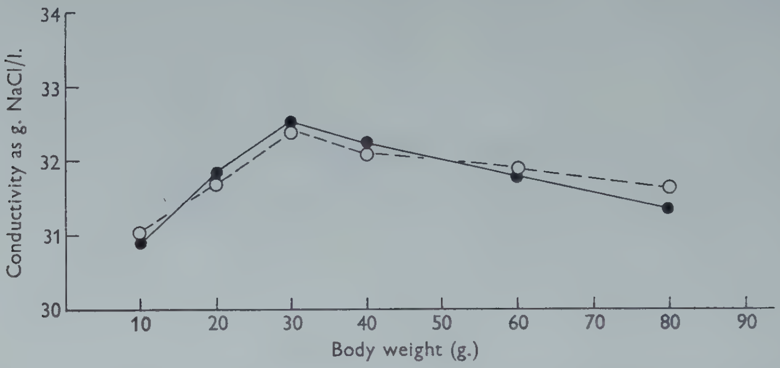


Fig. 4. Experimentally determined and calculated curves for body weight and conductivity: males only. ●—●, Experimental; ○- - -○, calculated.

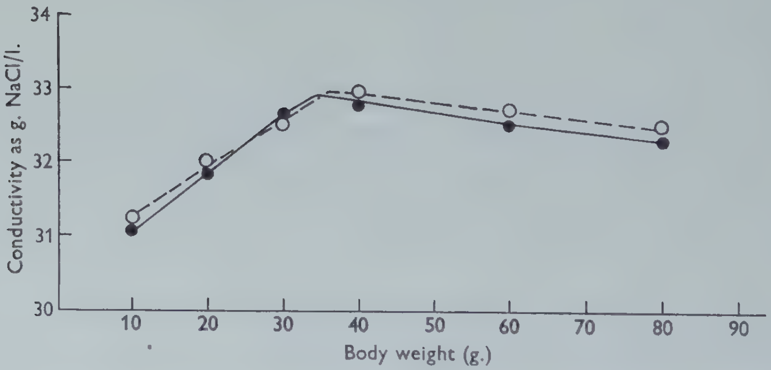


Fig. 5. Experimentally determined and calculated curves for body weight and conductivity: females only. ●—●, Experimental; ○- - ○, calculated.

Using Shaw's values for Na^+ K^+ Ca^{2+} Mg^{2+} for the composition of the blood and those of the present work for Cl^- and $SO_4{}^{2-}$ it is possible to estimate the total millimolar blood concentration for females of 35 g. body weight. Values for the total mM. ionic content are in close agreement with total mM. concentration as obtained from the freezing-point depression, and with the values for conductivity measurements. For this weight therefore blood concentration is apparently almost entirely due to electrolyte. If the relative proportion of the different ions remains constant over the whole size range the blood conductivity can be estimated: the estimated values for females are plotted, together with those determined experimentally in Fig. 4. Fig. 5 shows similar agreement for males, although there is more doubt about the validity of the assumption on which the estimates are based. Clearly the hypothesis that the relative proportion of the ions remains constant over the whole size range for both sexes appears to be justified.

At 35 g. body weight the total O.P. as shown by the freezing-point depression agrees well with the total O.P. as estimated from the conductivity and blood analysis of Shaw (1955) and Gilbert (1959). At other weights, however, there are considerable discrepancies. Since Figs. 4 and 5 show that it is highly probable that the relative proportion of the ions remains constant over the whole size range it follows that these discrepancies must be due to the non-electrolyte fraction; when the ions decrease this must increase and vice versa. Drihlon Courtois (1934) has already shown that under certain conditions blood protein increases as the ionic concentration decreases in individual crabs; an investigation of the non-electrolyte variation in crabs of different body size and sex will be reported in a future communication.

SUMMARY

1. The influence of sex and body weight on the concentration of sulphate and chloride ions in the blood of the common shore crab was investigated.

2. Blood chloride increased in both sexes until a maximum was reached at a weight of about 35 g.; thereafter it fell with increasing body weight.

3. Over the whole size range blood chloride was higher in females than in males. This difference was highly significant for animals over 35 g. body weight; below 35 g. however, there was no significant difference between the sexes.

4. Over a restricted size range blood sulphate of males was significantly higher than that of females.

5. Results of the present work have been discussed in relation to those reported earlier for conductivity and total O.P.

I am indebted to Prof. A. D. Hobson for his help and consideration at all times, and to Dr C. Ellenby for his unstinted encouragement and advice as well as for reading the manuscript. I also wish to express my thanks to Mr J. Shaw for his many suggestions and to the staff of the Dove Marine Biological Laboratory, Cullercoats.

REFERENCES

Bateman, J. B. (1933). Osmotic and ionic regulation in the shore crab, *Carcinus moenas*. *J. Exp. Biol.* **10**, 355–72.

Drihlon Courtois, A. (1934). De la régulation de la composition minérale de l'hémolymphe des crustacés. *Ann. Physiol.* **10**, 377–414.

Duval, M. (1925). Recherches physico-chimiques et physiologiques sur le milieu intérieur des animaux aquatiques. Modifications sous l'influence du milieu extérieur. *Ann. l'inst. Oceanogr. Monaco*, N.S., **2**, 233–403.

Gilbert, A. B. (1959). The composition of the blood of the shore crab *Carcinus moenas* Pennant in relation to sex and body size. 1. Blood conductivity and freezing-point depressions. *J. Exp. Biol.* **36**, 113–19.

NAGEL, H. (1934). Die aufgaben der Exkretion sorgane und der Keimen bei der osmoregulation von *Carcinus moenas*. *Z. vergl. Physiol.* **21**, 468–491.

ORTON, J. H. (1936). Experiments in the sea on rate of growth of some crustacean decapods. *J. Mar. Biol. Ass. U.K.* **20**, 673–89.

QUNITON, R. (1904). *L'eau de mer, milieu organique.* 2me éd. ch. 3. Paris.

ROBERTSON, J. D. & WEBB, D. A. (1939). The micro estimation of sodium, potassium, calcium, magnesium, chloride and sulphate in sea water and body fluids of marine animals. *J. Exp. Biol.* **16**, 155–77.

SENDROY, J. (1939). Micro determination of chloride with solid silver iodate. 1. Gasometric analysis. *J. Biol. Chem.* **120**, 335–403.

SHAW, J. (1955). Ionic regulation in the muscle fibres of *Carcinus moenas*. *J. Exp. Biol.* **32**, 383–96.

WEBB, D. A. (1940). Ionic regulation in *Carcinus moenas*. *Proc. Roy. Soc.* B, **129**, 107–35.

THE CUTICLE OF *ACARUS SIRO* L. (= *TYROGLYPHUS FARINAE*)

By T. E. HUGHES, M.A.
Zoology Department, Birkbeck College, London

(*Received* 9 *August* 1958)

INTRODUCTION

The cuticle of *Acarus siro* is of two distinct types. That of the appendages is similar in appearance to the sclerotized cuticle of many adult insects; and there is no reason to suppose that it differs from this at all. The cuticle covering the opisthosoma, however, is exceedingly thin and colourless, the dorsal plate having undergone extreme reduction. There is no visible distinction into endo- and exo-cuticle. The cuticle as a whole forms a thin stiff layer lying directly on the epidermis. It is not elastic and if mites are kept at low humidities grooves develop in the cuticle as the internal volume decreases by loss of water.

Since *Acarus siro* can be bred in large numbers it is a suitable animal for the investigation of the thin colourless cuticle of the opisthosoma.

METHODS

In order to obtain samples of cuticle, heavy cultures of *Acarus siro* were placed in funnels over water, in which the active instars of the mites were collected. This prevented the inclusion of any resting stages, which might contain untanned skeletal proteins. The mites so obtained were broken by slight grinding in a tissue homogenizer with a loose plunger. They were then digested at pH 7·2 with trypsin for 24 hr. The products of tryptic digestion were removed by repeated washing with distilled water. When samples of washings concentrated to 10% of their original volumes gave no ninhydrin reaction, it was considered that the residual material included only hardened skeletal fragments. Microscopic examination showed only empty pieces of exoskeleton and a few guanine (Hughes, 1950) excretory bodies.

The cuticular material thus obtained was analysed for various elements including nitrogen and sulphur. Some samples were hydrolysed by refluxing for 12 to 20 hr. with 5·0N hydrochloric acid or with 5·0N caustic soda solution under an atmosphere of nitrogen. Acid hydrolysates, after filtering, were evaporated to dryness under reduced pressure and then taken up in 10% isopropanol. Alkaline hydrolysates brought to neutrality were de-salted electrolytically. A flowing anode of 5% sulphuric acid and a cathode of mercury circulated by water were used. These hydrolysates were then analysed by paper chromatography. Other hydrolysates were analysed for nitrogen and sulphur. The chromatography was done on strips, or by two-way partition; some circular chromatographs were also made. The solvents used were water-saturated phenol and butanol-acetic-water 5:1:4.

Whole mites and cuticular material obtained as described were subjected to the chitosan test in order to determine the presence or absence of chitin in the skeleton. The quantities of material available were small, 0·2 g. of cuticle being the average value. In the present instance it has so far not been possible to attempt anything other than a qualitative analysis of this material. The smallness of the mites makes it impossible to obtain enough material of ecdysing stages to attempt an analysis of the untanned cuticular proteins.

RESULTS

1. The chitosan reaction is weakly positive except on the appendages where it is strongly positive (the skeleton here is thicker and includes hard brown sclerotin).

2. Quantitative analysis for elements:

Material	% Nitrogen	% Sulphur
Whole cuticle unhydrolysed	9·75–10	1·71–2·0
Acid hydrolysate, soluble fraction	6·0 – 6·5	0
Acid hydrolysate, insoluble humin	3·0 – 3·5	2·0
Alkaline hydrolysate	6·0 – 6·25	2·0

3. Table of amino acids identified:

Amino acid	Alkaline hydrolysate	Acid hydrolysate
Aspartic	++	++
Glutamic	++	++
Glycine	+++	+++
Serine	+	++
Threonine	–	Trace
Lysine	+	+
Arginine	–	+
Proline	+	+
Leucine	+	+
Valine	+	+
Tyrosine	+	+
Alanine	+++	+++

The depth of colour given in the ninhydrin reaction on development of the chromatographs has been indicated by + signs.

The acid hydrolysate also included glucosamine, and colorimetric estimation of phenols using Folin and Ciocalteu's reagent gave a ratio of

$$\frac{0{\cdot}6}{1{\cdot}0} = \frac{\text{Phenols of acid hydrolysate}}{\text{Phenols of alkaline hydrolysate}}.$$

Spectographic analysis of the hydrolysates showed absorption in the range 220–230 mμ with a plateau between 250 mμ and 270 mμ.

The acid hydrolysate produced a black humin. This was dissolved in cold 5·0N caustic soda solution, evaporated to dryness under reduced pressure and extracted with three successive lots of ethyl ether. The ether extract which was straw-coloured contained a substance which gave a positive reaction for sulphydryl groups with sodium nitroprusside and a blue colour with Folin and Ciocalteu's reagent before the application of ammonia, indicative of a paraphenolic group. A similar phenol occurs in the alkaline hydrolysate.

DISCUSSION

The weak chitosan reaction of the skeleton, apart from the appendages, is in agreement with the fact that on hydrolysis the glucosamine reaction is no stronger than that given by the majority of individual amino acids. This indicates that the amount of chitin in the skeleton is relatively small.

The results of the analysis for elements are of special interest in that they showed the presence of sulphur in far larger quantities than the results of similar analyses of insect cuticle (Hackman, 1953). From the examination of sections of mites over a period of years it had been thought from staining reactions that this thin cuticle might be a protein material similar to keratin. This view was strengthened by the report (Beament, 1951) that the egg shells of the red spider mite, *Metatetranychus ulmi*, contain sulphur, and are plasticized to some extent by agents which destroy di-sulphide linkages.

However, when the results of chromatography of hydrolysates were compared with those obtained by treating white wool in an identical manner, this idea was seen to be erroneous. The wool hydrolysates gave considerable quantities of cystine which was entirely lacking from the hydrolysates of mite cuticle.

The sulphur in the acid hydrolysate is confined to the humin, a black intractable residue. It is there in combined form as it cannot be extracted by sulphur solvents. Since a phenolic substance, probably a paraquinone, is extractable from the humin and since this same extract gives a sulphydryl reaction it is possible that the sulphur is present as SH groups attached to a quinone, probably a paraquinone. The phenolic substance obtained, when chromatographed on strips with butanol-acetic-water solvent, gives a spot of R.F. value 0·85. Dennell (personal communication and in press) obtained from insect cuticle (treated with alkaline stannite under anaerobic conditions) a substance which under similar chromatographic treatment gives a spot R.F. 0·83. This substance gives every indication of being a paraquinone with amino groups attached.

The presence of lysine, in both alkaline and acid hydrolysates, precludes the presence of the particular type of quinone-amino acid bonding postulated for insect cuticles by Pryor (1940*a*, *b*) and Pryor, Russell & Todd (1947). In the insect cuticle the ϵ-amino group of lysine plays an important part in the formation of cross links between the protein molecules by the aromatic nuclei, the other amino groups concerned being mainly terminal ones. It is, moreover, known that when there is competition between a sulphydryl and an amino group in reactions with quinones, there is a preference for the sulphydryl to react (James & Weissberger, 1939; Wittle *et al.* 1953). Thus, if the protein of this particular cuticle contained sulphydryl groups, it could be tanned by quinones forming cross links with them similar to those occurring in insect sclerotin between quinones and the amino groups of neighbouring molecules.

Acid hydrolysis of a protein tanned in this way through quinone linkage with sulphydryl groups could not produce any cystine as it does from wool. It might conceivably produce cysteine if the linkages between quinone and sulphydryl group were easily broken. An essential feature of tanned protein is, however, that the cross bonding is not easily broken. The occurrence of sulphur still combined in the humin is evidence of the firmness of the linkage in this case.

If the linkage of quinone to sulphydryl groups of the protein were broken between the sulphur and the protein molecule by hydrolysis, there should appear in the hydrolysate the hydroxy-acid corresponding to cysteine, namely serine. This amino acid was not found in hydrolysates of insect sclerotin by Hackman (1953, 2). However, it appears in both acid and alkaline hydrolysates of the mite cuticle.

In alkaline hydrolysis under nitrogen serine is found in quantities comparable to other amino acids, although serine is known to be destroyed by boiling with caustic alkalis (Block & Weiss, 1956). This can only mean that serine is being slowly liberated by alkaline hydrolysis from some other compound, and in sufficient quantities to make good the losses involved in the process during the time of hydrolysis, if this does not continue too long. Alkaline hydrolysis prolonged beyond 24 hours leads to the disappearance of serine. It is known that cysteine will react by its sulphydryl groups with paraquinone to give substitution products (Mason, 1955; Fieser, 1941; Burton & David, 1952). A substance which is probably a paraquinone is extractable from the humin of acid hydrolysis. It is, therefore, here suggested, as a possible explanation of the experimental data, that in these mites the tanning process is basically the same as in insects, namely that enzymatically a quinone is produced from tyrosine or some other amino acid which contains a phenolic residue, and that this quinone then tans the protein produced by the epidermal cells by reaction with sulphydryl groups.

The essential difference between the typical hard sclerotin of insects and the thin white cuticle of these mites appears to be that, whereas the protein produced by the insect epidermis contains no sulphydryl groups, that of the mite does. Such a mechanism as is here postulated might well account for the sharp transitions from one type of cuticle to another, which are of frequent occurrence in arthropods. A basic tanning mechanism reacting with the epidermally produced proteins of cuticle might well be expected to produce different types of tanned cuticle, dependent on the type of protein with which it reacted.

It is not impossible that the aromatic nucleus of phenylalanine should provide the paraquinone nucleus (Dennell, 1958), and thus effect direct linkage between neighbouring protein molecules. Although tyrosine is present in both types of hydrolysate, no phenylalanine is found. Alanine is the amino acid giving the most marked ninhydrin reaction, equalled only by glycine.

Apart from the data supporting the suggestion that the cuticle is tanned through sulphydryl groups reacting with quinones, other differences from insect cuticle occur. No arginine or proline was obtained by Hackman (1953) from insect cuticles. These hydrolysates of *Acarus siro* cuticle include, of course, material from the legs and mouthparts which are pigmented. There is no reason to suppose that these yellow-brown parts are not hardened with sclerotin in the same way as in insects. Thus it would appear that amino acids not present in insect sclerotin must come from the thin cuticle of the opisthosoma. The fact that hydrolysis of many samples, prepared in the way described, always yielded the same amino acids, rules out the occurrence of serine and lysine as contaminants. In the case of contamination one would expect to find other amino acids too, and to get variable analyses.

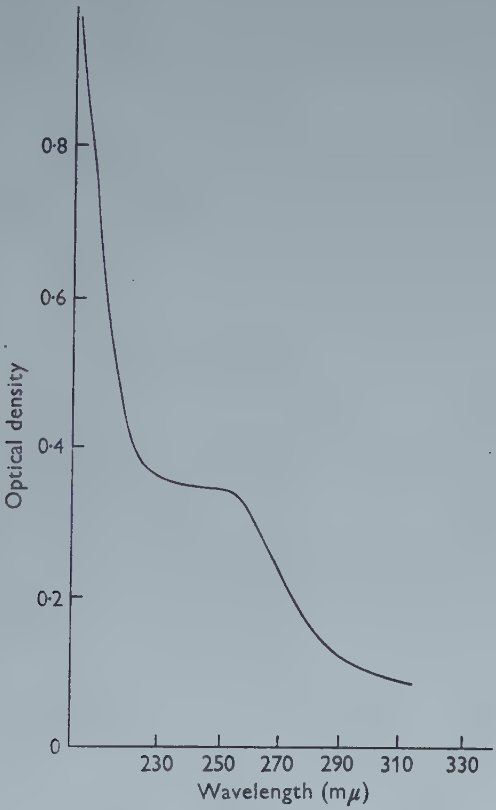


Fig. 1. Absorption spectrum of cuticular hydrolysates.

The estimation of phenolic substances gave results consistent with the hypothesis put forward here. The acid hydrolysate contains much less phenolic material than the alkaline one. This is to be expected since the humin includes phenolic material possibly combined with sulphur, and alkaline hydrolysis produces no such humin.

The light-absorption of the hydrolysates (Fig. 1) arises from the presence of phenols and it is interesting to note the close agreement of this curve with those obtained by Hackman (1953, 3, fig. 2) for cuticular protein tanned with or without the addition of catechol.

SUMMARY

1. The thin cuticle of *Acarus siro* contains a relatively large quantity of sulphur.

2. The sulphur does not form disulphide linkages between cysteine molecules as it does in keratin.

3. The presence of lysine amongst the products of hydrolysis shows that this cuticle is not tanned by linkage of quinone and amino groups.

4. The experimental data support the view that the cuticle is tanned by reaction of quinones and sulphydryl groups.

5. It is suggested that the quinone involved is a paraquinone, probably derived from phenylalanine.

ACKNOWLEDGEMENTS

I should like to thank Mr B. Cohen for independent verification of my chromatographic analyses and Dr R. Shaw for help and advice on absorption spectrum measurements. I should also like to thank Professor R. Dennell for discussion of details of his work on insect cuticle.

REFERENCES

BEAMENT, J. W. L. (1951). The structure and formation of the egg of the fruit tree red spider mite *Metatetranychus ulmi* Koch. *Ann. Appl. Biol.* **38**, 1–24.

BLOCK, R. J. & WEISS, K. W. (1956). *Amino acid handbook.* Illinois, U.S.A.

BURTON, H. & DAVID, S. B. (1952). Addition reactions of quinones. Pt. 1. The reaction of Cysteine and Thiourea and its derivatives with some quinones. *J. Chem. Soc.* pp. 2193–6.

DENNELL, R. (1958). The amino acid metabolism of a developing insect cuticle: the larval cuticle and puparium of *Calliphora vomitoria*. The non-specific hydroxylation of aromatic amino acids and the production of polyphenols by the cuticle. *Proc. Roy. Soc.* B, **148**, 280–4.

FIESER, L. F. (1941). Chemistry of Vitamin K. *Ann. Intern. Med.* **15**, 648–58.

HACKMAN, R. H. (1953). The chemistry of insect cuticle. 1. Water soluble proteins. 2. Water insoluble proteins. 3. Hardening and darkening of the cuticle. *Biochem. J.* **54**, 362–77.

HUGHES, T. E. (1950). The physiology of the alimentary canal of *Tyroglyphus farinae*. *Quart. J. Micr. Sc.* **91**, 45–61.

JAMES, T. H. & WEISSBERGER, A. (1939). Oxidation processes. XIII. Inhibitory action of sulphite and other compounds on the autoxidation of hydroquinone and its homologues. *J. Amer. Chem. Soc.* **61**, 442–53.

MASON, H. S. (1955). Comparative biochemistry of the phenolase complex, pp. 105–84. *Advances in Enzymology and Related Subjects of Biochemistry*, vol. 16. New York.

PRYOR, M. (1940*a*). On the hardening of the ootheca of *Blatta orientalis*. *Proc. Roy. Soc.* B, **128**, 378–93.

PRYOR, M. (1940*b*). On the hardening of the cuticle in insects. *Proc. Roy. Soc.* B, **128**, 393–407.

PRYOR, M., RUSSELL, P. & TODD, A. R. (1947). Phenolic substances concerned in hardening the insect cuticle. *Nature, Lond.*, **159**, 399.

WITTLE, E. L. *et al.* (1953). The synthesis of Pantetheine–Pantethine. *J. Amer. Chem. Soc.* **75**, 1694–700.

THE RESPONSES OF SNAILS TO POLARIZED LIGHT*†

By EDWARD R. BAYLOR

Woods Hole Oceanographic Institution, Woods Hole, Mass.

(*Received* 21 *November* 1958)

The purpose of this paper is to call attention to the use of extra-ocular polarization analysis in the polarized light behaviour of a snail and to suggest that an extra-ocular polarization analyser could be employed by a number of organisms reported to orient to polarized light. Behaviour responses to polarized light have been reported for a number of arthropods: Verkhovskaya (1940), von Frisch (1949), Pardi & Papi (1953), Kerz (1950), Wellington, Sullivan & Green (1951), Wellington (1953, 1955), Stevens, Fingerman & Brown (1953), Baylor & Smith (1953, 1957*a*, *b*), and Vowles (1954*a*, *b*). The behaviour responses of a marine snail, *Littorina littorea*, to polarized light have been reported by Burdon-Jones & Charles (1958). The diversity of dioptric systems suggests that more than one mechanism of polarization analysis may be involved or that some ubiquitous polarizing analyser not directly associated with the dioptric system may exist.

Intra-ocular polarizing analysers suggested so far depend on the existence of 'Polaroid'-like (dichroic) structures as proposed by Autrum & Stumpf (1950); or they depend on a single reflexion-refraction at the air-corneal interface at the polarizing angle according to Stevens *et al.* (1953); or they depend on two separate double reflexion-refraction events at the corneal surface as suggested by Baylor & Smith (1953).

One extra-ocular polarizing analyser suggested by Baylor & Smith (1957*b*) for the honey bee proposes a non-uniform distribution of intensities in the pattern of light reflected from a substratum illuminated by polarized light. Vertical parallel rays of polarized light illuminating a substratum give rise to reflexions with minimum intensities in a direction parallel to the plane of polarization and maximum intensities perpendicular to the plane of polarization. But vertical parallel rays of non-polarized light illuminating a substratum give rise to reflexions of uniform intensities in all directions. In the escape reaction of the bee, orientation to polarized light occurs only if the bee is permitted to see the substratum and it occurs whether or not the bee is permitted to see the overhead source of polarized light illuminating the substratum. It remains to be seen whether the communication dances of bees described by von Frisch are mediated by extra-ocular polarization analysis or by some intra-ocular device. Indeed, in view of the non-uniform distribution of intensities reflected from appropriate substrata illuminated by polarized light it is possible that any positive phototaxis or even a light-directed klinokinesis can appear to be an ability to discriminate the plane of polarization.

* Contribution no. 996 from the Woods Hole Oceanographic Institution.

† Aided by a grant from National Institutes of Health.

Additionally, two other extra-ocular polarizing analysers may be available to organisms orienting to polarized light under water. Clay particles, yeast, bacteria, or phytoplankton suspended in water scatter light with greater intensity at right angles to the plane of polarization thereby providing an asymmetric intensity stimulus. The importance of using carefully filtered water for polarized light experiments is obvious. Compounds such as toluidine blue, tryptophane, riboflavine, and chlorophyll A fluoresce strongly. When these compounds are excited by polarized light fluorescence is emitted perpendicular to the plane of polarization. Such asymmetric fields of intensity produce orientation to polarized light in snails on non-analysing substrata.

METHODS AND RESULTS

The marine snail, *Nassa obsoleta*, employed in these experiments possesses a simple positive phototaxis like that of *Littorina littorea*. Both bearing and distance travelled are easily measured and provide an adequate description of the behaviour.

We did the following experiments to test whether *Nassa obsoleta* has a polarizing analyser in the eye or utilizes the pattern of intensity gradients reflected from the substratum. The snails were allowed to crawl on a substratum of black 'Lucite' illuminated by a vertical beam of light which also projected a Cartesian co-ordinate grid upon the substratum. The lines of the grid were extremely thin and did not interfere with the behaviour to be measured. Each time the snail crossed a line the event was counted. Thus the pathway of the animal is measured as the sum of all the X vectors and the sum of all the Y vectors. If orientation is random, the path lengths in the X and Y directions should be approximately equal to each other. In control experiments the light source was non-polarized. In experiments with polarized light the plane of polarization was used alternatively parallel to the X axis and to the Y axis so as to obviate any inherent bias in the system. The polished black 'Lucite' substratum showed no differential reflected intensity in non-polarized light but showed a slight differential reflected intensity in polarized light.

The results of control experiments using non-polarized light on black 'Lucite' show random orientation. In Fig. 1 the path lengths in the X and Y directions are plotted against each other. The points are distributed on both sides of the diagonal line which indicates randomness of orientation.

In contrast, results of experiments using polarized light on polished black 'Lucite' show definite orientation perpendicular to the plane of polarization. In Fig. 2, the path length perpendicular to the plate of polarization is plotted against the path length parallel to the plane of polarization. With few exceptions all the points lie above the diagonal.

In order to determine whether the organism could respond to substratum reflectivity, scratched 'Lucite' was used. The scratches on the black 'Lucite' were all parallel and threw the surface into tiny ridges and valleys. Non-polarized light striking the sides of the ridges is reflected predominantly at right angles to the direction of the scratches. Polarized light vibrating parallel to the direction of the scratches is well reflected at right angles but poorly reflected parallel to the direction of the scratches. Polarized light vibrating perpendicular to the direction of the scratches is poorly reflected perpendicular to the direction of the scratches, but is even more poorly reflected in the direction of the scratches.

Regardless of the plane of polarization of light the snails uniformly oriented perpendicular to the scratches. The data in Fig. 3 show that orientation was most pronounced when the plane of polarization was parallel to the scratches.

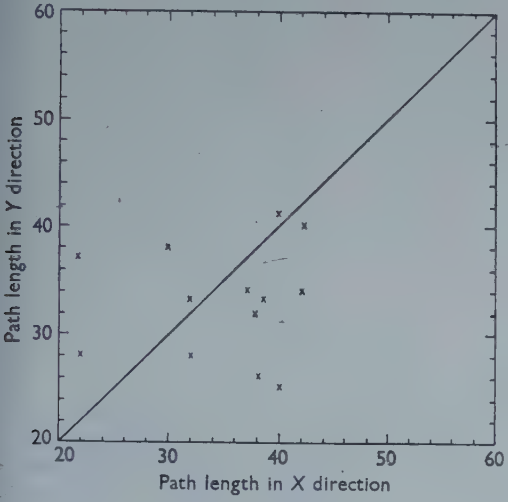


Fig. 1

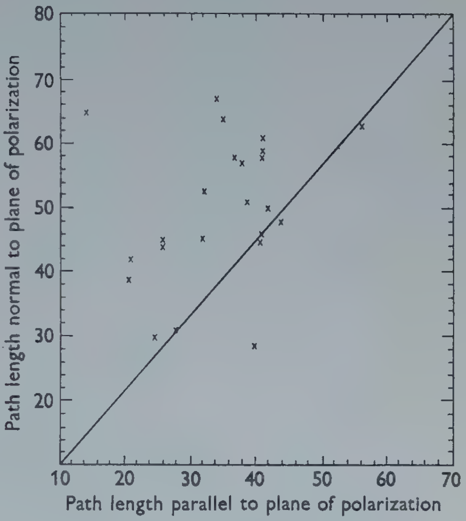


Fig. 2

Fig. 1. Orientation to non-polarized light by snails crawling on polished black 'Lucite'. Path lengths of the snails were measured and plotted on a Cartesian co-ordinate grid. When points fall equally on both sides of the diagonal line orientation is random.

Fig. 2. Orientation to polarized light by snails crawling on polished black 'Lucite'. The diagonal line represents random orientation. Orientation is predominantly at right angles to the plane of polarization.

To eliminate the possibility of snails seeing the overhead source of polarized light we attached black 'umbrellas' to the shell. The 'umbrella' was a circle of 'Scotch' photographic masking tape 10 to 20 mm. in diameter completely shading the tentacles bearing the eyes. A small area of the shell was scraped clean for attachment of the 'umbrella'. It is important that the attached 'umbrella' does not seriously alter the centre of gravity of the snail.

Snails carrying black 'umbrellas' in polarized light on polished black 'Lucite' oriented perpendicular to the plane of polarization although they could not see the incident radiation. The data from Fig. 4 show that this response was obtained in all but three experiments.

To eliminate the possibility of snails seeing the substratum and to assure their seeing only the overhead source of polarized light we caused them to crawl upside down underneath a plate of clear glass. The plate of glass was 25 mm. above the substratum. The snails carried their 'umbrellas' beneath them. It was necessary

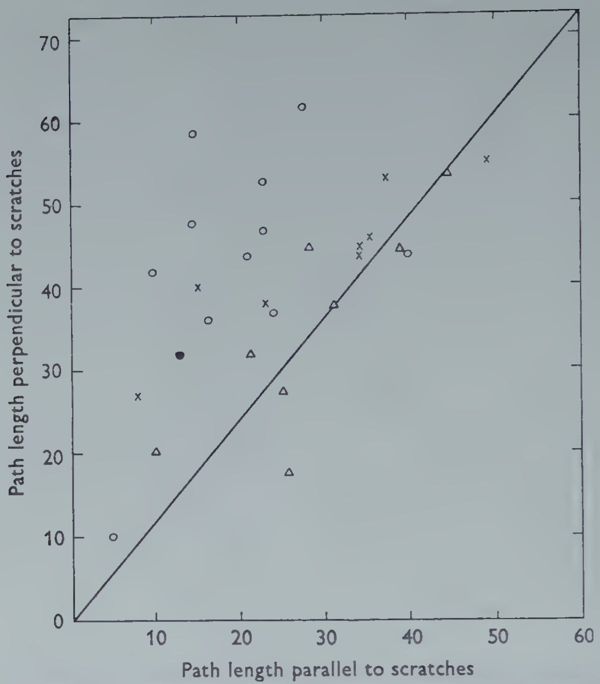


Fig. 3. Orientation of snails on scratched black 'Lucite'. Orientation to polarized light appears maximal when the plane of polarization is parallel to the scratches on the substratum. This combination of plane of polarization and direction of scratches produces maximum reflected intensity perpendicular to the scratches. The triangles are the path length in non-polarized light. Circles are path length when the plane of polarization is parallel to the scratches. Crosses are path lengths when the plane of polarization is perpendicular to the scratches.

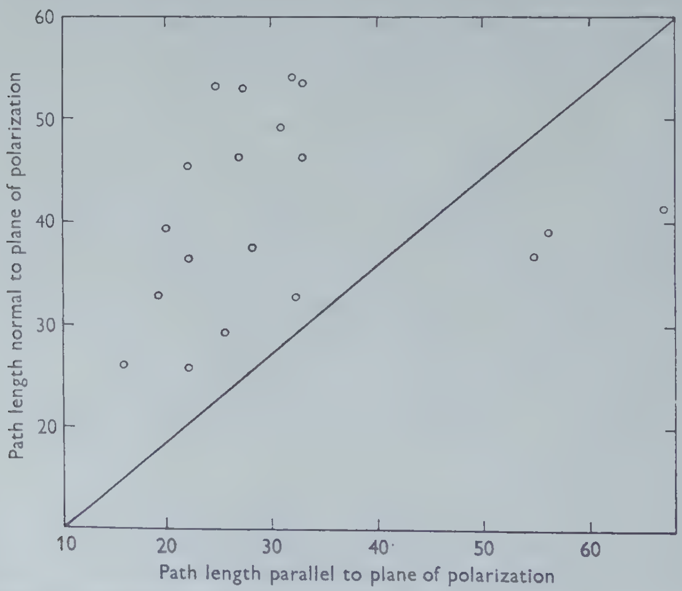


Fig. 4. Orientation to polarized light by snails carrying black 'umbrellas' while crawling upright on polished black 'Lucite'.

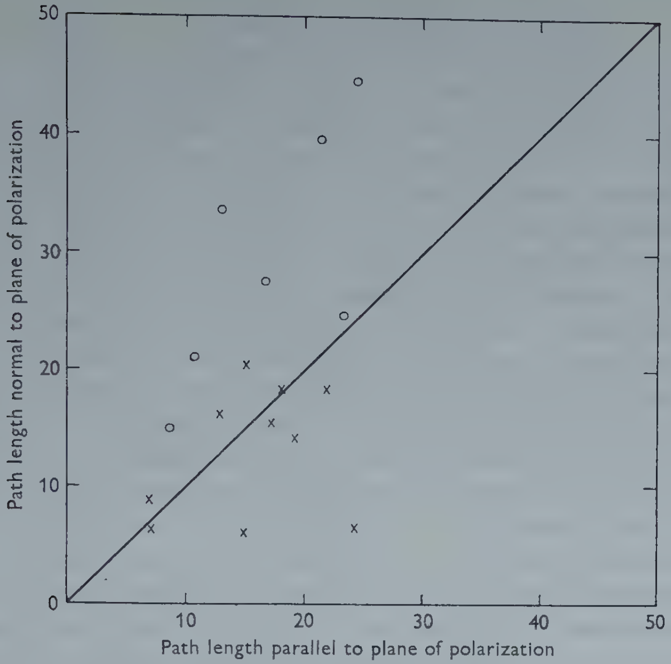


Fig. 5. Orientation to polarized light by snails crawling upside down with and without 'umbrellas' beneath them. Crosses show path lengths of snails with 'umbrellas' beneath them permitting no view of the polished black 'Lucite' substratum underneath but permitting a direct view of the polarized light source above. Circles show path lengths of snails without 'umbrellas' beneath them, permitting a view of both the substratum underneath and the polarized light source above. Orientation to polarized light appears best in snails permitted to see the substratum.

to attach a small float to prevent the snail from falling off the glass plate. No orientation was apparent in these experiments, shown in Fig. 5, although the overhead source of polarized light is fully visible to the snails. Control snails, in polarized light, crawling upside down and without 'umbrellas' orient almost as well as the snails crawling upright.

DISCUSSION

The conclusion from these experiments is that *Nassa obsoleta* under polarized light with all spurious reflexions reduced to a minimum is oriented by the intensity of substratum reflexions and not by a polarizing analyser within the eye. Also it follows from these experiments that the mere exhibition of an oriented response to polarized light does not inevitably imply the possession of a polarizing analyser within the eye.

An organism using extra-ocular polarization analysis orients to asymmetric directions of intensity or patterns of intensity which arise by: (1) preferential reflexion from the substratum; or by (2) preferential scatter from suspended particles such as yeast, algae, bacteria, clay, etc.; or by (3) preferential emission of fluorescence perpendicular to the plane of polarization of the exciting light in compounds like chlorophyll *a*, riboflavin, and chlorophyll degradation products.

So far extra-ocular polarization analysis appears to be principally a laboratory phenomenon. Its use by animals in the natural habitat is probably limited to situations where the sun but not all the sky is obscured from view, as for example in the shadow of a cliff, under trees, or in other conditions obscuring the sun. As a rule the normal intensity of light in the terrestrial environment is so much greater than the reflected intensity from polarized light that extra-ocular polarization analysis is not anticipated.

Three different mechanisms have been proposed as possible intra-ocular polarizing analysers. Autrum & Stumpf (1950) have suggested that the rhabdomeres of the honey bee are dichroic like 'Polaroid' and that they constitute more efficient receptors of polarized light than of non-polarized light. Stephens *et al.* (1953) have proposed that in accordance with the Fresnel laws a single reflexion-refraction at the corneal surface of the *Drosophila* eye serves as a polarization analyser. Baylor & Smith (1953) independently advanced a reflexion-refraction mechanism for the cladoceran and acarinid eye which involves a somewhat unusual light path having two reflexions and refractions.

The Autrum & Stumpf (1950) hypothesis of intra-ocular polarization analysis is intuitively appealing and suggests a model which is consistent with the experimental observations of von Frisch (1950) on the communication dances of bees. But actual proof of the model as a valid analogue to the eye is lacking. Menzer & Stockhammer (1951) reported birefringence of bee rhabdomeres, but they used fixed material for their observations. Using unfixed material, de Vries *et al.* (1953) finds that the birefringence, if it exists, of the bee rhabdomere is less than 1 %. The principal experimental evidence advanced by Autrum & Stumpf (1950) in favour of their model was a contention that the insect compound eye is a more efficient receptor of polarized light. This contention was based on a slightly greater magnitude electroretinogram voltage associated with polarized light stimulation than with non-polarized light stimulation. They employed a 50 % neutral density filter to give non-polarized light of the same intensity as polarized light. Thus the increased electroretinogram voltage with polarized light in their experiments can be anticipated from the fact that most 'Polaroids' are not good neutral density filters. On the contrary, depending on the conditions of manufacture, they leak at one end of the visible spectrum or at both ends. When a light source is viewed through such polaroids crossed exactly at 90 degrees to each other the light source appears blue, red, or purple. Baylor & Kennedy (1958) using better intensity and wavelength control failed to corroborate the results of Autrum & Stumpf (1950) and adduced other evidence against a dichroic model of the bee eye. Further, extra-ocular polarization analysis has been demonstrated in the escape reaction of the honey bee by Baylor & Smith (1957*b*). Their findings, which are consistent with the experimental observations of von Frisch (1950), suggests that the communication dances of the bee are 'cued' by extra-ocular polarization analysis. Although their interpretation remains to be proved, it provides a more parsimonious explanation of von Frisch's observations than the Autrum & Stumpf model.

It is quite feasible for such organisms as *Cladocera*, having both an intra-ocular polarizing analyser and apositive phototaxis, to utilize both intra- and extra-ocular polarization analysis simultaneously. It is not clear at this time how much substratum reflexion contributes to the polarized light responses of *Cladocera* but the polarized light response persists when substratum reflexions, fluorescence, and suspended particle scatter are eliminated.

In addition to substratum reflexion, *Cladocera* utilize the asymmetric intensity of scattered light found when a suspension of yeast or algae is illuminated by polarized light. Similarly, the asymmetric intensity of fluorescence of riboflavin, toluidine blue, chlorophyll degradation products, etc., excited by polarized light appears to enhance the response of daphnids.

There is no good evidence available at this time for cladoceran orientation to the polarized light of the sky.

It is unfortunate that some papers in the literature strongly imply that because there is a response to polarized light, it therefore must have evolutionary adaptive value as a navigational aid. Any timidity in pressing this classical fallacy of teleology is understandable. The invocation of polarized light as a navigational aid is probably unnecessary because usually when the sky is strongly polarized the sun is visible and inference of solar azimuth by polarized light becomes redundant. Pre-sunrise and post-sunset skies may constitute important exceptions and although both starlight and 'Gegenschein' are polarized there is as yet no necessity to invoke the polarization of either as an explanation of animal behaviour.

In concluding this discussion it seems worth while to suggest that most instances of polarized light response reported in the literature should be re-examined to determine whether they are 'cued' by intra-ocular or extra-ocular polarization analysis.

SUMMARY

1. Experiments were performed to test whether the polarized light responses of the marine snail, *Nassa obsoleta*, are mediated by a polarizing analyser within the eye or by the pattern of intensities reflected from the substratum.

2. Snails able to see only the substratum orient at right angles to the plane of vibration of vertically incident polarized light.

3. Snails unable to see the substratum but able to see the overhead source of polarized light fail to orient to the polarized light.

4. Implications regarding the rare occurrence of intra-ocular polarizing analysers and the improbability of extensive animal use of polarized light as a navigational aid are discussed.

REFERENCES

Autrum, H. & Stumpf, H. (1950). Das Bienenauge als Analysator für polarisiertes Licht. *Z. Naturf.* **5*b***, 118–112.

Baylor, E. R. & Smith, F. E. (1953). The orientation of *Cladocera* to polarized light. *Amer. Nat.* **87**, 97–101.

Baylor, E. R. & Smith, F. E. (1957*a*). Diurnal migration of plankton crustaceans. *Recent Advances in Invertebrate Physiology*, 21–36. University of Oregon Press.

Baylor, E. R. & Smith, F. E. (1957*b*). Bees and polarized light. *Symposium on Pattern Recognition* 1957 (ed. W. P. Tanner, Jr). Ann Arbor, Michigan: Project Michigan Press.

BAYLOR, E. R. & KENNEDY, D. (1958). Evidence against a polarizing analyzer in the bee eye (in the Press).

BURDON-JONES, C. & CHARLES, G. H. (1958). Light reactions of littoral gastropods. *Nature, Lond.*, **81**, 129–31.

DE VRIES, H. L., SPOOR, A. & JIELOF, R. (1953). Properties of the eye with respect to polarized light. *Physica*, **19**, 419–32.

FRISCH, K. VON (1949). Die Polarisation des Himmelslichtes als orientierender Faktor bei den Tänzen der Bienen. *Experientia*, **5**, 142–8.

FRISCH, K. VON (1950). Die Sonne als Kompass im Leben der Bienen. *Experientia*, **6**, 210–21.

KERZ, M. (1950). Wahrnehmung polarisierten Lichtes durch *Eupagurus*. *Experentia*, **6**, 427.

MENZER, G. & STOCKHAMMER, K. (1951). Zur Polarisationsoptik der Facettenaugen von Insekten. *Naturwissenschaften*, **38**, 190.

PARDI, L. & PAPI, F. (1953). Richerche sull'orientatamento di *Talitrus saltator* (Montagu) (Crustacea—Amphipoda). II. *Z. vergl. Physiol.* **35**, 490–518.

STEPHENS, GROVER C., FINGERMAN, M., & BROWN, F. A. (1953). The orientation of Drosophila to plane polarized light. *Ann. Ent. Soc. Amer.* **46**, 75–83.

VERKHOVSKAYA, I. (1940). L'influence de la lumière polarisée sur la phototaxis de quelques organismes. *Bull. Soc. Nat. Moscou* (Sect. Biol.), **49**, 101–13.

VOWLES, D. M. (1954*a*). The orientation of ants. I. The substitution of stimuli. *J. Exp. Biol.* **31**, 341–54.

VOWLES, D. M. (1954*b*). The orientation of ants. II. Orientation to light, gravity, and polarized light. *J. Exp. Biol.* **31**, 356–75.

WELLINGTON, W. G., SULLIVAN, C. R. & GREEN, G. W. (1951). Polarized light and body temperature level as orientation factors in the light reactions of some hymenopterous and lepidopterous larvae. *Canad. J. Zool.* **29**, 339–51.

WELLINGTON, W. G. (1953). Motor responses evoked by the dorsal ocelli of Sarcophaga aldrichi Parker, and the orientation of the fly to plane polarized light. *Nature, Lond.*, **172**, 1177.

WELLINGTON, W. G. (1955). Solar heat and plane polarized light versus the light compass reaction in the orientation of insects on the ground. *Ann. Ent. Soc. Amer.* **48**, 67–76.

INTERACTION BETWEEN WALKING AND PROBING IN *APHIS FABAE* SCOP.

By A. IBBOTSON* and J. S. KENNEDY
Agricultural Research Council Unit of Insect Physiology,
Zoology Department, University of Cambridge

(*Received* 8 *January* 1959)

INTRODUCTION

In a previous paper (Ibbotson & Kennedy, 1951) it was shown that the clustered distribution of the black bean aphid, *Aphis fabae* Scopoli, on its host plants was due partly to the gregariousness of the aphids. In a preliminary analysis of the stimuli mediating this behaviour the distribution of probes made by aphids walking on leaf surfaces was examined. Probes were grouped close to stationary live aphids, close to black models of aphids, where the divergence of a side vein impeded progress along a main vein, and where the aphids first encountered the leaf surface on debouching from a card 'cat-walk' up which they had climbed. Probes made close to other aphids were of longer duration than those made close to inert models, at the divergence of side veins or elsewhere on the leaf.

The act of probing is an elementary behaviour pattern universal among aphids whether they are gregarious or not. Further work was concerned primarily with the probe-evoking stimuli provided by the aphids themselves; but inevitably it touched upon other factors governing probing but not immediately concerned in aggregation. These experiments having a more general bearing are considered below, and the rest in a separate paper (Ibbotson, in preparation).

In order to avoid the irregularities of a leaf surface which themselves elicited probes, most of these experiments were done with aphids off the plant entirely. Typically, the aphids were made to walk along uniform cat-walks of card or paraffin wax about 2 mm. wide. These simulated leaf veins in that an aphid, once astride such a ridge, had a tendency to continue walking along it. Each experimental arrangement was enclosed in a white muslin tent set directly under a 60 W. tungsten lamp 45 cm. above the test aphids, so that they were uniformly illuminated and the observer sitting outside the tent was concealed from them. Further details of methods are given in the appropriate sections below. Only apterous virginoparous adults were used, bred in the mass culture described by Kennedy & Booth (1950) and selected as detailed below.

---

* Now at: School of Agriculture, King's College, Newcastle-upon-Tyne.

RESULTS

The act of probing

The series of movements making up a probe on the inert surfaces was the same as on a leaf (Ibbotson & Kennedy, 1951). The aphid checked its forward movement, protracted its rostrum, and, with the antennae pointing forwards and still vibrating, made a number of decisive 'jabs' at the surface beneath it. The legs were then disposed for centripetal traction and the antennae laid back as the aphid finally came to rest. Sometimes the rostrum was half raised for a short distance before the aphid stopped completely; occasionally, when the foothold seemed uneven, the aphid moved over the substrate in a more or less crab-wise movement, trailing the tip of the rostrum along the surface beneath it before jabbing; on yet other occasions the jabbing movements were interrupted and the aphid moved to a fresh position before completing the probe.

When the aphid's stylets penetrate deeply the labium becomes shortened and elbowed as the head approaches the surface. Aphids were commonly seen in this condition on the plants in the culture and could then be induced to withdraw their stylets only by prolonged stimulation. If such an aphid was forcibly dislodged the stylets were pulled out of the plant tissue and were obvious to the naked eye. On wax surfaces deep insertion of the stylets did not seem to occur as the rostrum was not elbowed, and when the aphid was dislodged the stylets were not similarly visible. Slight penetration by the stylets may have occurred because probes made on wax lasted longer, before locomotion was resumed, than they did on glass into which penetration was of course impossible. Moericke (1950) noted that *Myzus persicae* (Sulz.) left a minute drop of saliva at the point of each probe made on glass.

Elicitation of probes

Aphids kept in a glass container for some hours in the dark were found to be stationary with the rostrum retracted against the body. They began protracting the rostrum and probing only on exposure to light, which soon induced walking instead. On the other hand, when they were walking any considerable restraint imposed upon their progression led sooner or later to probing in situations which differed widely as far as the type of sensory input was concerned.

(1) *Total restraint of locomotion*

Aphids were taken at random from among those walking on the cage walls of the mass culture and each was suspended by a wire attached to the dorsum with spirit gum. When held without tarsal contact they waved their legs irregularly in the manner which usually leads to the righting of a free aphid fallen on its back. The leg movements were spasmodic, and combined irregularly with them were protractions and retractions of the rostrum. As the leg movements gradually subsided the aphid often came to rest with the rostrum protracted (Table 1).

When the tarsi were given contact with solid surfaces of four different kinds these were gripped by the claws or plantulae (the inflatable adhesive sacs at the tibiotarsal joint) so that the leg movements now took a locomotory form, with prolonged straining in one direction or another, until this again gradually ceased. Protraction of the rostrum rarely occurred in this situation until the phase of leg activity was over, apparently as the result of fatigue, and the preliminary jabbing with the rostrum seen in free aphids was never observed. When the aphids came finally to rest the rostrum was protracted about equally often on the four different surfaces, as with no tarsal contact at all (Table 1).

Table 1. *Numbers of suspended aphids, out of five per trial, which had protracted the rostrum into the probing position after 1 hr.*

Tarsi in contact with	Trial no.					Total out of 25
	1	2	3	4	5	
Air only	1	2	2	3	2	10
Filter-paper	3	2	3	2	3	13
Muslin	1	2	3	3	2	11
Glass	3	2	2	2	3	12
Wax	2	3	2	2	1	10

(2) *Texture of the substrate*

As reported in the previous paper, aphids walking astride a cardboard cat-walk often probed when they passed from this on to a leaf. Other 'neutral' surfaces were tested in the same way. Twin cardboard cat-walks each 2 mm. wide were arranged to lead almost vertically upwards, each to the centre of a circular test surface 2 cm. in diameter pressed against a window in a flat metal plate. In order to magnify the differences in the quality of the foot-hold that these surfaces provided, the plates were inclined over the cat-walks so that the aphids on the test surfaces were supporting their own weight in an upside-down position. Young adults taken from the walls of the culture cages were starved in glass tubes for 24 hr. before testing. They were made to walk one at a time up the cat-walks on to each surface in turn, the order being reversed from aphid to aphid.

Table 2. *Numbers of aphids (out of n) that probed when walking upside-down across various flat surfaces*

n	Filter-paper		Clean glass	Smooth wax	Leaf (*Vicia faba*)
	Rough	Rough waxed			
10	1	3	—	—	—
10	1	—	9	—	—
20	0	—	—	—	18
20	—	1	—	11	—
20	—	—	4	11	—
20	—	—	6	14	—
20	—	—	—	11	12
20	—	—	—	14	15

On rough filter-paper and rough wax the aphids obtained a secure grip with their claws and continued to move forward freely as on the cat-walk, and very few probes were made (Table 2). On the glass and still more on the smooth wax the aphid's tarsal claws seldom engaged and the plantulae were brought into play giving a poor grip against gravity. Some aphids lost all foothold and fell and were discarded. The progress of the others was halting and clumsy, for obtaining and releasing each foothold took longer with the plantulae than with the claws, and probes often ensued. Probes occurred on smooth wax almost as often as on a host leaf.

These results may be contrasted with those obtained from tethered aphids on horizontal surfaces of diverse texture as above. The tethered aphids gained a sufficient grip on all those surfaces to be stimulated by the resistance they offered (due to the tethering) into making strong locomotory traction responses with the legs. The eventual inhibition of the locomotor reflexes that was accompanied by rostrum protraction could reasonably be attributed to fatigue. There was little resistance to traction for free aphids upside-down on the surfaces offering an insecure grip, and the leg strokes were curtailed. These aphids often protracted the rostrum and began jabbing before leg movements had ceased, and fatigue alone could hardly account for the final complete inhibition of locomotory movements as probing proper began. Here it appeared to be the onset of probing itself which completed the process of inhibition of the locomotory reflexes begun by the substrate.

(3) *Form of the substrate*

Some substrates stimulated locomotory movements at the expense of probing. On a vertical cardboard strip, the width of which exactly fitted the span of the legs, the aphid grasped the two edges with the tarsal claws, advanced rapidly and undeviatingly when illuminated from the dorsal side, and probed very rarely. On a strip which was either too wide or too narrow for its edges to be gripped symmetrically the aphid's progress was irregular and slower, but leg movements were nevertheless strongly stimulated by the good grip obtained by some of the tarsi. The aphids made frequent changes of posture and grip, sometimes lurching over one edge and even sidling round to the opposite unlit face of the strip. Probes were more frequent than on a strip of the correct width, but still infrequent compared with those made on wax and glass where the claws seldom engaged. The elicitation of this 'locomotor struggling' at the expense of probing recalls the behaviour of the aphids tethered on various surfaces (see above) before fatigue reduced their locomotory excitation.

With wax it was possible to compare uniform substrates with the same, correct, width but different shapes. Three 2 mm. ridges were made by drawing metal templates along blocks of paraffin wax 10 cm. long (Fig. 1). A model aphid (black stone) was fixed at one end of each ridge as a standard probe-evoking object. In two duplicate experiments twenty-five fresh young adults were set to walk along each horizontal ridge in turn toward the model. When the ridge was square in cross-section (Fig. 1 *a*) the aphids gripped its two edges firmly with the claws, walked rapidly from one end to the other, and climbed over the model without probing at all (Table 3). When the ridge was almost flat (*c*) they advanced stably with moderate speed but did not maintain a straight course and usually left the ridge before reaching the model. When the ridge was smoothly semicircular in section (*b*) they stayed on it to the end but moved forward more slowly and waveringly, and more than half of them probed when they reached the model. In addition to the probes made in the immediate vicinity of the model, a number of the aphids probed elsewhere along this semi-circular ridge. These probes were not localized but distributed at random, like the probes made intermittently on the waxed Petri-dish rim (see below).

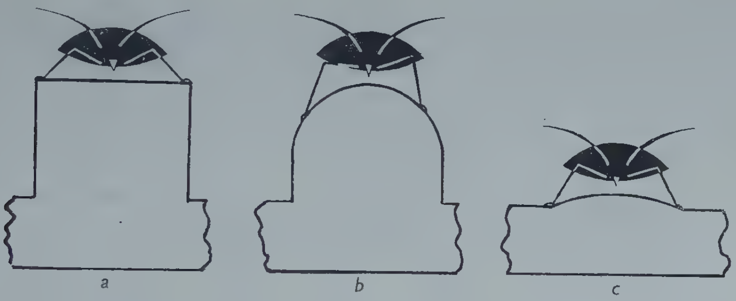


Fig. 1. Diagrammatic frontal view of aphids walking along wax ridges of equal width but different cross-sections, affecting the quality of foothold and gait (see text).

Table 3. *Numbers of probes made within* 5 *mm. of model aphids set at the far end of straight wax ridges* 10 *cm. long, of different cross-section* (*see Fig.* 1); *and numbers of probes made at random along each ridge*

(Two batches of twenty-five aphids, each aphid walking individually along each ridge in turn.)

Situation of probes	Shape of ridge in cross-section		
	Almost flat (*c*)	Semi-circular (*b*)	Square (*a*)
Localized, within 5 mm. of model	0	12	0
	0	17	0
At random	0	9	1
	2	5	0

When watched through a medium-power binocular microscope, the aphids walking along the square ridge were seen to make firm contact with the tarsi at every step, after about the same amount of leg extension, and this even length of stride set up a regular walking rhythm which carried the aphid forward at a good pace. On the semicircular ridge the forward-swinging tarsi made contact with the ridge again at varying levels up and down its curved sides, so that the amount of leg extension was irregular from step to step and leg to leg. The aphid lurched slightly from side to side, continually making compensatory changes in the amount of leg extension and flexion in such a way as to keep the body oriented more or less along the ridge. That the aphids on this ridge were being repeatedly disoriented but correcting their orientation in this manner was confirmed by removing the overhead light. They then walked steadily along the square ridge as before but wandered off the semicircular one after a few seconds. Stimuli from the semicircular ridge were not symmetrical enough to hold the aphids astride it without the addition of further symmetrical stimulation from the overhead light, although the former stimuli had more orienting effect than those from the ridge that was almost flat. The locomotory movements of the aphids on the semicircular ridge suffered, in the result, more impediment than on either of the other two ridges.

(4) *Individual objects*

The relation between probes made at random along the uniform ridges and what we may call 'localized' or simply 'local' probes made in the immediate vicinity of newly encountered objects was examined further by setting aphids to walk round the waxed rim of a 9 cm. Petri dish (Fig. 2). The rim was coated by repeated dipping in molten paraffin wax and then smoothed until semicircular in cross-section and about 2 mm. wide. The dish was surrounded by a 5 cm. high wall of cartridge paper 2·5 cm. away, all within the white muslin tent. In this situation the aphids remained walking round the rim for long periods. Five of them left there overnight on one occasion were still walking around it next morning. If the overhead light was turned off, or the perimeter wall or muslin sides were removed, thus destroying the uniformity of the visual field, the aphids soon climbed down from the rim.

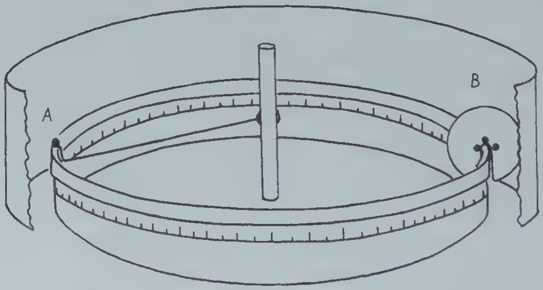


Fig. 2. Sketch of Petri dish with waxed rim, and (cut away) perimeter wall, showing arrangement of live aphid fixed on wire (*A*) and transparent, black-spotted disk (*B*) used for testing probing responses of aphids walking round the rim.

The first objects used to provide localized stimulation for aphids walking on the rim were other live aphids attached with spirit gum by the ventral surface of the abdomen to the flattened ends of thin copper wire, arranged as shown in Fig. 2*A*. Walking aphids that began to climb over one of these fixed ones, or caught up with the walking aphid next in front, were immediately lifted over with a capillary suction tube applied to the dorsum and set down on the rim beyond, where they continued walking. By this means five aphids could be kept circling the rim and encountering the fixed objects with only rare encounters among themselves. A batch of five aphids was taken from the culture on three separate occasions, and allowed to circle the rim together until each had completed five circuits in three consecutive trials: (i) with one live aphid fixed on the rim, (ii) with two live aphids fixed at diametrically opposite points on the rim, and (iii) with none. The three trials were conducted in a different order with each batch, and the distribution of probes was recorded by reference to a millimetre scale beneath the rim.

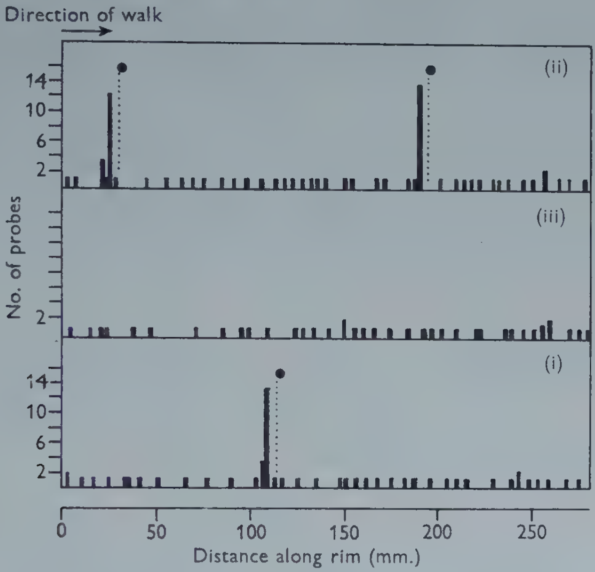


Fig. 3. Distribution of probes made by aphids walking round waxed Petri-dish rims and encountering one, two, and no live aphids fixed upon it at the positions indicated by black circles and dotted lines. Total random probes made more than 5 mm. from a fixed aphid: 36 with arrangement (i), 37 with (ii) and 38 with (iii).

The total number of probes made at random round the rim was about the same in (i), (ii) and (iii), although the total number of local probes varied with the number of fixed aphids present (Fig. 3). The number of local probes made within 5 mm. of each fixed live aphid was about the same in (i) and (ii). Within the limits of the experiment, readiness to stop and probe was thus unaffected by the total distance walked since last encountering an object, or by the number of probes recently made. These experiments also showed that the probe-evoking effects of two objects at opposite points on the rim could be regarded as independent and thus compared.

The first objects compared by this means were fresh, young aphids (smooth, glossy and dark green) and older ones (rugose, matt and nearly black), both collected while wandering in the culture cages. Free aphids of these same two age-groups were tested in batches of five against the two ages of fixed live aphids. Table 4 shows that young walking aphids were more responsive than older ones and that fixed young aphids evoked fewer responses than did fixed old ones.

Table 4. *Numbers of localized and random probes made per twenty-five circuits of the circular waxed rim by successive batches of five old or five young test aphids, when similar old or young aphids were fixed at opposite points on the rim*

Free aphids under test	Localized probes		Random probes
	At fixed, live old aphid	At fixed, live young aphid	
Old	3, 1, 0, 0	0, 0, 1, 0	2, 0, 0, 0
Young	14, 11, 10, 10	7, 4, 6, 3	4, 4, 0, 0

Further comparisons were designed to separate and identify those features of a fixed live aphid which stimulate another approaching aphid to stop and probe. Objects embodying particular aphid-like features, and control objects resembling them except for the lack of the chosen features, were both tested against the common standard of a live older aphid fixed on the opposite side of the rim. The objects are listed below:

Aphid-simulating	Controls
1. Aphid model (visual): black stone of aphid size	1*a*. White stone of aphid size
2. Aphid group silhouette: transparent disk of talc 2 cm. in diameter set astride the rim by a radial slot, and painted with 3 black spots 2 mm. diam. and 2 mm. apart grouped round the apex of the slot on the rim (Fig. 2)	2*a*. Transparent talc disk without black spots
3. Aphid odour: white (stone) model smeared with the fresh reddish brown wax extruded from the siphunculi of adult aphids as a reflex response to rough handling—in this case pinching the thorax with forceps	3*a*. White aphid models smeared (i) with a pigment suspension simulating the appearance of the models smeared with siphuncle wax, and (ii) with honeydew
4. Mechanical impediment: a loose meshwork of cotton fibres lying across the rim	4*a*. Three horse-shoe-shaped copper wires 0·5 mm. diam. and *c*. 2 mm. apart, lying across rim
5. Mechanical block: a thin glass rod drawn to a point and set above and parallel to the rim in such a way that the point struck the walking aphid squarely on the frons	(5*a*. = 2*a*, i.e. less abrupt block)

Each object was tested with three separate batches of five free aphids on the rim. Tests were conducted only with those batches which, in a preliminary check, made ten or more local probes in every twenty-five encounters with the fixed aphid. At this level of responsiveness the free aphids differentiated clearly between each object which embodied a chosen aphid-like feature and the control object which lacked it, consistently making ten or more local probes in every twenty-five encounters with the former, and less than five in every twenty-five encounters with the latter.

Aphids approaching the black model (1) and the siphuncle-wax-smeared model (3) stopped abruptly and probed at once, usually just before their forward-pointing antennae had touched it. They reacted to the broader group silhouette (2) in the same way except that some aphids moved crab-wise on the rim with the rostrum protracted either before or during jabbing. The failure of the control objects 1*a*, 2*a* and 3*a* showed that the visual and olfactory stimuli given by other aphids were both effective in causing walking aphids to stop and probe, while honeydew was not. Mechanical obstruction was most immediately effective when encountered in the most sudden and complete form, as the glass point (5). This usually passed between the forward-pointing antennae without touching either and the impact it then made on the frons induced a temporary complete inhibition of leg movements. On the other hand, the mechanical obstruction provided by the cotton fibres (4), which interrupted the leg movements directly and occasionally caught the claws, was not effective until after a few seconds, and the wires (4*a*) over which the aphids clambered with the minimum of constraint were ineffective with aphids at the given level of responsiveness.

Effects of pre-treatment on probing

In addition to the variation in responsiveness mentioned above as associated with age in aphids taken from the walls of the culture cages in the glasshouse, where they were free to leave and return to the plants, some of the variation among these aphids appeared to be governed by the weather in the preceding hours. In selecting the batches of five aphids for testing, as above, it was found that those collected and tested in the morning usually responded well to probe-evoking objects on the rim; whereas if the day had been bright and warm since the early hours, aphids of similar appearance were relatively unresponsive. Similarly, those collected and tested in the afternoon usually responded poorly, but if the day had been particularly dull and cool they often responded well. These weather differences also affected the amount of wandering seen in the cages. When bright, warm conditions prevailed all day wandering activity showed one peak in the earlier part of the morning and a second peak in the late afternoon, with a lull around midday when most of the morning wanderers were back on the plants. This sequence recalls the diurnal variations in the aerial population of alate aphids (Johnson, 1954). In dull weather there was less wandering altogether and little sign of a midday lull. Prior activity off the plant was thus associated with lesser readiness to probe on encountering an aphid-like object. These effects were investigated further as follows.

Aphids were reared on young bean plants in two dark rooms maintained at 19 and 24° C. Within 24 hr. of the last moult the adult apterae were separated into batches of 50 and given one of the treatments listed in Table 5. Aphids from each treatment were then tested in turn on a waxed Petri dish rim in the same two dark rooms, with two diametrically placed transparent talc disks astride the rim, one with and the other without three black spots (objects 2 and 2*a* in the previous experiment). Each treatment was applied to two batches of aphids and timed so that the second batch was ready for testing the day after the first. All the treatments, and the series of probing trials, were started at about 9 a.m. each day. Each trial lasted about 30 min. so that the batch tested last in a given day's series had been undergoing its treatment for several hours longer than the batch that was tested first. The different treatments were equalized in this respect by testing in a different

Table 5. *Mean numbers of localized probes near the talc disks, and of random probes, made per twenty-five circuits of the circular waxed rim, by four separate batches of test aphids from each treatment*

(All treatments except E carried out in the dark. Starved aphids kept in a clean Petri dish. All experiments conducted after the aphids had been exposed to the light for 15 min. at the temperature at which the experiment was done.)

Treatment	Localized probes		Random probes
	At spotted disk	At plain disk	
At 19° C.			
A. Fresh from rearing plants	18·00	6·25	8·25
B. Starved for 24 hr.	13·00	3·75	21·50
C. Starved for 24 hr., tested at 24° C.	8·25	2·00	17·75
D. Starved for 48 hr.	4·50	0·25	48·25
E. Starved for 24 hr., in daylight	7·00	1·00	14·50
F. On young bean shoots for 48 hr.	24·25	23·75	34·25
At 24° C.			
G. Fresh from rearing plants	17·00	6·00	9·25
H. Starved for 24 hr.	11·75	2·75	45·50
J. Starved for 24 hr., tested at 19° C.	20·00	8·00	30·00

Analysis of Variance

	S.S.	D.F.	M.S.	*F*
Total	17643·55	107	—	—
Probes	6937·85	2	3468·92	466·19
Treatments	4006·46	8	500·81	67·30
I.A.	6096·49	16	381·03	51·21
Error	602·72	81	7·44	—

Minimum significant difference between treatment means within probe type: at 5 %, 6·18; at 1 %, 7·25.

order each day: 1st day—A, B, C, E, G, H, J; 2nd day—D, F, J, H, G, E, C, B, A; 3rd day—F, D.

Two sets of five aphids were taken at random in succession from each treatment-batch each day and set to walk round the rim as described above until each single aphid had completed five circuits. The total number of random probes, and of local probes made within 5 mm. of each talc disk, were recorded separately for each set. The mean numbers of probes per set (25 circuits) are given in Table 5 with a simple analysis of variance. The within-probe between-treatment minimum significant differences were computed by Tukey's method for comparison of treatment means (Snedecor, 1956).

Assuming that as fresh adults all batches entered treatment in the same physiological condition, the effect of the treatments was to produce batches of aphids in an almost continuous series of degrees of readiness to probe. There was a linear relationship between the number of local probes at the spotted disk and the number at the plain disk, with roughly three probes at the former for every one at the latter, except with the most responsive batch (F) which made equal numbers of probes at the two disks. The treatments did not, however, always affect local and random probing in the same way. The frequency of random probing was increased in varying degree by all the treatments, whereas local probes were sometimes increased and sometimes reduced by comparison with the behaviour of fresh aphids.

Keeping the aphids in the dark off the plant in a glass dish (where they apparently moved little and were found motionless at the end of the treatment) increased the frequency of random probes and at the same time reduced that of local probes, more or less in proportion to the duration of treatment or the temperature during treatment (A *v.* B *v.* D; G *v.* H; B *v.* H). A similar treatment in daylight where the aphids remained active (E) also increased random probing but significantly less than did darkness, and it reduced local probing still more. (Two batches given a tenth treatment not listed in Table 5, starvation in the dark at 24° C. for 48 hr., were all dead at the end of it.) 48 hr. spent at rest feeding and larvipositing on a fresh plant in the dark increased random probing less than did 48 hr. off the plant in the dark; and at the same time it increased local probes to the maximum instead of reducing time (F *v.* D). Transfer from a treatment temperature of 19° C. to a temperature of 24° C. for testing reduced random and local probes alike but not significantly (C *v.* B). On the other hand, transfer from a treatment temperature of 24° C. to a temperature of 19° C. for testing reduced random probing while substantially increasing local probes (J *v.* H). To sum up, keeping the aphids off a host plant lowered their threshold for locomotion-inhibiting stimuli from the substrate, but at the same time raised their threshold for stimuli received from other, incidental, aphid-like objects encountered while walking. The latter threshold was low in the fresh aphids which had long been subject, and responding, to stimuli from the host plant during their development, and it was lowered further only by continuing that same treatment.

DISCUSSION

So long as these parasitic insects are active but not on a host plant their activities are limited almost entirely to two motor patterns, walking and probing. As a rule only one of these occurs at one time. This is not simply because they are mechanically incompatible, for they occasionally overlap to some extent as when protraction of the rostrum and jabbing began before locomotion had ceased and when suspended aphids waved their legs and protracted the rostrum simultaneously. The mutual exclusiveness is evidently a central nervous interaction in the first place; the two alternative reflex systems are antagonists, excitation of either inhibiting the other. The clearest indication from the present work that they are antagonists in the full sense of Sherrington (1947) was the sustained alternation between them that was generated in the aphids by a particular external environment which was not itself alternating but constant. When an aphid was walking on the wax rim and lit from above, and the external inhibitory influence of the substrate upon locomotion reached a certain level, probing as it were 'broke through' (in Sherrington's phrase) the inhibitory influence which was being exerted upon it centrally by the locomotory activity in progress. The substrate soon inhibited probing in its turn and locomotion then broke through the central inhibitory influence of probing upon it; and so on, cyclically.

In alate virginoparous *A. fabae* a similar alternation generated by a constant environment has been found between locomotion (here flying) and probing, as reported preliminarily by Kennedy (1958). This phenomenon, and other features of the relation between the alata's two systems of reflexes, serving locomotion on the one hand and host exploitation on the other, showed a detailed parallelism with the relation between antagonistic reflexes in the mammalian spinal cord (Sherrington, 1947). Either of the two reflex systems could be caused experimentally to 'rebound', through 'successive induction' by the other. The present experiments were not designed to test for rebound in the aptera, but Moericke (1950) has demonstrated something very like it, successive colour contrast, in probing by both alate and apterous *Myzus persicae* (Sulz.).

Given an antagonistic relationship between two reflex systems, it becomes difficult to determine from simple behavioural experiments whether an observed response has been brought about by direct excitation, or indirectly by first inhibiting the antagonistic system. There is in fact no evidence that any of the external stimuli used in these experiments stimulated probing directly. Very diverse types of stimulation evoked probing, and inhibition of locomotion may have been their one common feature.

When the stimulation arose from direct mechanical interference with the normal pattern of locomotory reflex actions it gave a strong impression of causing probing in that indirect way, especially since the more abrupt and complete the arrest of locomotion the more promptly probing broke through. The most effective form of mechanical obstruction, in causing probing, was the glass point. Without any 'warning' from the antennae (nor apparently from other distance receptors) this suddenly arrested the forward swing of the body pivoting on all the legs, and thus presumably created the same kind of proprioceptive input as obstructing the legs themselves all at once. A summation in time of such inhibitory stimulation of locomotion was indicated by the longer delay before probing broke through when the cotton fibres did obstruct the legs themselves but not all at once; or when the transparent disk barrier was encountered first by the antennae, or when the uniform substrate alone hindered stepping.

Even when the stimulation that evoked probing was not mechanically obstructive but took effect through distance receptors, visual or olfactory, it may have acted through inhibition of locomotion; and direct evidence that visual stimulation from black objects can induce probing by this means will be given in the subsequent paper. No matter by what central pathway they act, the visual and olfactory stimuli that induced probing would contribute most to gregarious behaviour, because these types of stimulation are the least likely to be provided by objects other than fellow aphids in nature. If there are any non-host stimuli that evoke probing by exciting it directly, these more or less aphid-specific ones seem the most likely to do so, as a logical consequence of the evolution of the gregarious habit. But with present methods no more can be said on this point.

A further consequence of the antagonistic relationship between walking and probing is that the results of the pre-treatment experiment cannot be interpreted with any assurance in terms of the 'priming' of either activity. It seems reasonable to suppose that the excitabilities of walking and probing were both affected, in varying degree, by the different pre-treatments; but any distinction is difficult since any change in either would have some reciprocal effect on the other. Some clue is perhaps available from the paradoxical results of the treatments in which the aphids were denied access to a plant. All these treatments made the aphids probe more frequently at random along the ridge but less frequently when they encountered barriers across it. The increase in random probes implies some priming of probing (feeding 'centre') and the decrease of localized probes at barriers implies just the opposite, priming of locomotion. The results can be explained by assuming priming of both activities but not of either alone.

Thus in the alata (Kennedy, 1958) locomotory excitation, as measured by the rate of climb of the flying aphid, rose to a peak after each take-off following inhibition of probing on a non-host surface, and then declined more or less steadily. We may postulate that the same kind of sequence occurred during each bout of walking by the apterae between the random probes which they made on the wax ridge. Assuming also that the priming of locomotion raised the peak level of locomotory excitation attained within each walking bout, then locomotion-inhibiting stimuli encountered around the peak in each bout would become less effective, leading to the observed decrease in the number of probes at the obstacles. The priming of probing could nevertheless show itself in causing probing to break through sooner as locomotory excitation was declining again, thus reducing the average duration of each walking bout and leading to the observed increase in the frequency of random probes. Increased flight vigour combined with decreasing duration of flight bouts was recorded in the alata.

SUMMARY

1. In experiments with apterous adult aphids walking on non-plant surfaces, rostrum protraction and probing of the surface were elicited by a variety of situations having the common effect of interfering mechanically with the normal pattern of locomotory movements.

2. A uniform wax ridge which fitted the span of the aphid's legs but afforded a poor grip to the tarsi and broke up the stepping rhythm, combined with an overhead light, generated a cyclical alternation between walking and probing.

3. When the uniform substrate was interrupted by some form of obstacle to locomotion further probes were elicited, and the more abruptly and completely locomotion was arrested, the more promptly these appeared.

4. Visual stimulation (from black models of aphid size) and olfactory stimulation (from the aphids' siphuncle wax) also caused walking aphids to stop and probe.

5. Readiness to probe varied greatly with age and pretreatment. Aphids denied access to a host plant for 1–2 days probed more frequently at random in response to the substrate but less frequently at objects encountered.

6. Walking and probing interact in the manner of antagonistic reflex systems, and the diverse types of stimulus which elicited probing may all have done so indirectly by first inhibiting locomotion.

This work was done while the first author held a scholarship from the Agricultural Research Council, and his thanks are due to Prof. V. B. Wigglesworth for granting him facilities with the Unit of Insect Physiology.

REFERENCES

IBBOTSON, A. & KENNEDY, J. S. (1951). Aggregation in *Aphis fabae* Scop. Aggregation on plants. *Ann. Appl. Biol.* **38**, 65–78.

JOHNSON, C. G. (1954). Aphid migration in relation to weather. *Biol. Rev.* **29**, 268–85.

KENNEDY, J. S. (1958). The experimental analysis of aphid behaviour and its bearing on current theories of instinct. *Proc. 10th int. Congr. Ent., Montreal*, 1956, no. 2, 397–404.

KENNEDY, J. S. & BOOTH, C. O. (1950). Methods for mass rearing and investigating the host relations of *Aphis fabae* Scopoli. *Ann. Appl. Biol.* **37**, 451–70.

MOERICKE, V. (1950). Über das Farbsehen der Pfirsichblattlaus (*Myzodes persicae* Sulz.). *Z. Tierpsych.* **7**, 265–74.

SHERRINGTON, C. S. (1947). *The Integrative Action of the Nervous System*, 2nd ed. London: Cambridge University Press.

SNEDECOR, G. W. (1956). *Statistical Methods*, 5th ed. Ames, Iowa: Collegiate Press.

THE WATERPROOFING MECHANISM OF ARTHROPODS

I. THE EFFECT OF TEMPERATURE ON CUTICLE PERMEABILITY IN TERRESTRIAL INSECTS AND TICKS

By J. W. L. BEAMENT

Agricultural Research Council Unit of Insect Physiology,
Department of Zoology, University of Cambridge

(*Received* 17 *January* 1959)

INTRODUCTION

In a recent paper Beament (1958*a*) has examined the physico-chemical problems associated with the measurement of evaporation from small animals and has described a method of investigation which gives results suitable for physical analysis; this method reveals an abrupt breakdown in the waterproofing system of the cockroach, *Periplaneta*, at a critical temperature. An analysis of the experimental data suggests that the unusually low permeability of the cockroach cuticle to water is due to an organized monolayer of grease and that this becomes disorientated at a transition temperature. Beament's paper demonstrates that a phenomenon of importance can be masked in the results obtained by simpler experimental methods. Although simple methods have revealed an apparent resemblance between the cockroach and certain other insects in respect of this property, we cannot necessarily conclude that other insects besides cockroaches have a transition phenomenon in their temperature-permeability relationship. There are important differences between the epicuticular structures of the few insects which have been examined in any detail; in particular, the cockroach has a waterproof grease (Beament, 1955), whereas many terrestrial arthropods appear to rely on hard waxes for waterproofing (Beament, 1945; Wigglesworth, 1945; Lees, 1947; etc.). A selection of terrestrial arthropods has now been investigated. A slightly modified version of the equipment previously described (Beament, 1958*a*) has been used; this is described in the appendix, and photographs appear in Beament (1958*b*). From measurements on the rate of evaporation at various temperatures, before and after the application of adsorptive dusts, it is possible to discern in more detail the form which the waterproofing system may take in some of these insects.

Adjectival nouns in the phrases 'skin temperature', 'air temperature', etc., will be used throughout; in particular 'air-cuticle temperature difference' means the difference in temperature between the surface of an animal and the surrounding air at such distances from the animal that air temperature is determined only by thermostatting equipment. 'Permeability/temperature curve' will be used for the plot of the permeability of a cuticle at constant saturation deficiency against cuticle temperature. The layer of wax secreted on to the epicuticle surface at the time of ecdysis will be called the 'primary wax layer'.

INITIAL EXPERIMENTS

For the initial experiments the adult male of the locust, *Schistocerca gregaria*, was selected. The experimental procedure of Beament (1958*a*) was followed, but accurate readings could be taken after much shorter time intervals of desiccation because the new balance had much greater sensitivity. For an increment of a few degrees in temperature stable conditions could be obtained some 5–10 min. from the time of adjusting the temperature control, and readings of evaporative loss were taken every minute at each stable temperature until three successive readings agreed. A complete temperature/permeability curve could thus be constructed from experiments involving a very small loss of the water contained in the specimen. Dry air was flushed through the box after each resetting of the temperature control, and also whenever the accumulated water of evaporation inside the box amounted to 1% relative humidity. Information was obtained in order of increasing temperature (if the temperature is lowered during a series of observations the technique must be modified (p. 398)).

Fig. 1, curve *A*, shows the result of such an experiment on an adult locust; permeability is expressed at unit saturation deficiency gradient for various surface temperatures. The curve does not contain a sharply defined discontinuity, though the permeability of the cuticle is changing markedly with temperature and rises steeply at temperatures above 48° C. Comparing this with corresponding measurements on the cockroach (Beament, 1958*a*) we could conclude that the locust—which appears to have a hard wax on its surface—does not show a transition temperature and that we have no evidence for a special waterproofing system based on an organized monolayer of wax. But an insect which has been anaesthetized in 5% carbon dioxide in air and then killed by exposure to hydrogen sulphide gas has many of its spiracles fixed in the open position. When a living locust is ventilating its tracheal system the water loss through its spiracles is appreciable, and though no information has been found on corresponding losses from dead insects the results shown in curve *A* will certainly include some evaporative loss through the spiracles. It seemed possible that the curve for cuticular transpiration alone might still contain a discontinuity if the effect of tracheal evaporation were removed, and the experiments were therefore repeated with animals in which the spiracles had been blocked. Dr T. Weis-Fogh kindly provided specimens in which he had occluded the spiracles with wax.

Fig. 1, curve *B*, shows the result of determinations made on such an animal, which was so far as possible identical in size and weight with that used for curve *A*. Against all expectations, the rate of evaporation at all temperatures was found to be greater than from the corresponding specimen whose spiracles were open; curve *B* is also smoother and even less likely to contain a discontinuity than is curve *A*.

These experiments were repeated a number of times, and wax, paint or injected liquid was used to block the spiracles. A family of curves was obtained, each showing the property of an individual. The only general conclusion which could be reached was that the rate of water loss is lower at all temperatures if the spiracles are not touched. Though considerable variation was found from one animal to another, and though the most waterproof of the animals with blocked spiracles lost water less rapidly (at all temperatures) than the most permeable of the untreated animals, it was obvious that the least waterproof animal gave the smoothest curve.

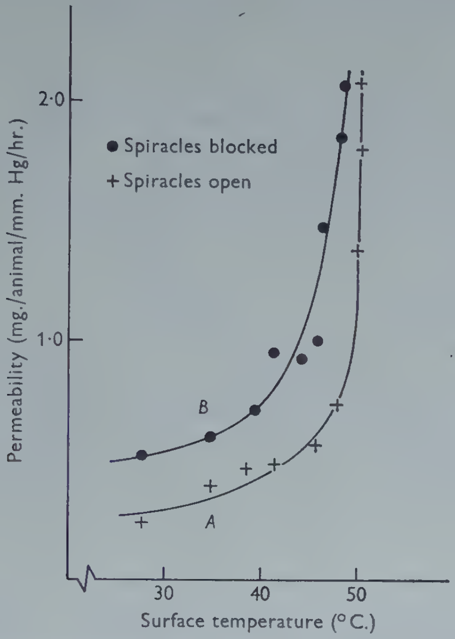


Fig. 1. Graph showing the permeability of the cuticle of the adult locust *Schistocerca gregaria* at various cuticle temperatures. *A*, an animal with some spiracles open; *B*, an animal whose spiracles have been occluded.

Similar experiments were performed on large nymphs of *Rhodnius*; and again animals with blocked spiracles showed, in general, higher rates of water loss and smoother permeability/temperature curves. But the best of the untreated animals did give a curve with a sharp break—of the kind clearly suggesting a transition point—at about 60° C. It was then found that a control experiment, in which a *Rhodnius* was handled as though to block the spiracles but in which the spiracles were left untouched, revealed high water loss and a very smooth curve. In contrast, animals which had been carefully captured, killed and transferred to the balance without having been touched by the operator's hand all gave sharp breaks in their permeability/temperature curves and were very waterproof. This series of initial experiments concluded with the demonstration of a sharp discontinuity in the curve for an adult locust when the specimen had likewise been manipulated in the most careful manner possible.

The author has not so far found any method of blocking spiracles which does not involve appreciable damage to the waterproofing system, and in the results which are here reported the apparent permeabilities must include some component due to evaporation from the tracheal system. Failure to separate the two sources of water loss has to some extent limited the accuracy with which the data can be analysed, but the discovery of the extreme delicacy of the waterproofing system has proved of much greater importance (p. 410); further, some of the divergence between the measurements made by previous authors and those described here may well be attributed to the damage incidental to blocking spiracles.

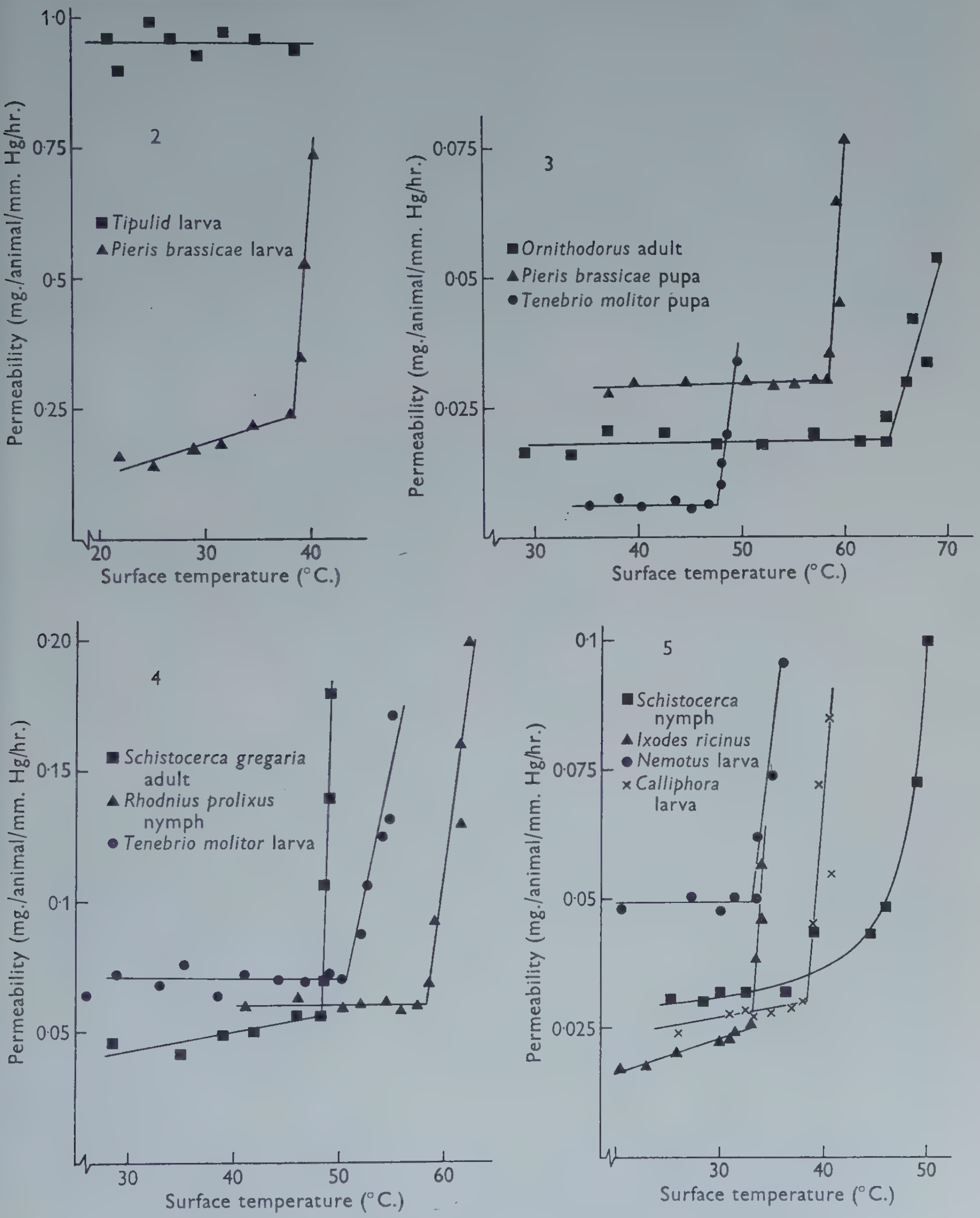
EXPERIMENTAL PROCEDURE

The animals used in subsequent experiments were selected, wherever possible, at an appropriate stage of their life history, just previous to moulting. They were transferred to clean glass containers and allowed to moult there; where possible they were also killed in these containers and transferred to the balance in ways which involved the least possible damage. In particular, the pupa of *Pieris* was selected for much of the latter work in this study because it could be manipulated entirely by its silk. We must, however, bear in mind, when considering the actual rates of water loss recorded below, that insects in nature may have sustained superficial damage to their cuticles. Wigglesworth (1945) shows that a soil-living larva of *Agriotes* has an extremely permeable cuticle, though the cuticle is very much more waterproof if the insect is allowed to moult away from the soil. He attributed this state of affairs to gross abrasion of the waterproof layer; the degree of damage caused by handling insects in the present type of experiment is probably very much smaller.

To obtain a curve (such as those of Figs. 2–5), the properties of at least four individuals of the closest equivalence in age, instar, past history, etc., were examined. The first animal of each group was used to establish the approximate form of the permeability/temperature curve, measurements being taken over short periods of time at temperature intervals of 5° C. This experiment showed the region of temperature likely to contain a sudden change in permeability, and made it possible to assess the order of water loss in relation to the total water content of the specimen. Each of the remaining individuals was then examined more critically; the range of temperature likely to contain a discontinuity was covered in small steps and the duration of desiccation was adjusted according to the depletion of the specimen's water content. In principle it is essential that each curve should be obtained from one individual (Beament, 1958*a*), but in fact the three individuals of each group showed only small differences in the form of the curve, and the characteristics of the particular individuals selected for Figs. 2–5 are probably representative of that species at that stage of its life history. Individual variation within the species has not been investigated here in greater detail, but changes caused by the ageing of the cuticle are shown in detail later (p. 403).

RESULTS

From Figs. 2 to 5 it is clear that all but two of the permeability/temperature curves contain sharp discontinuities; they are so clearly defined that they obviously represent the sudden breakdown in waterproofing envisaged by Wigglesworth (1945)



Figs. 2–5. Graphs showing the permeability to water of the cuticles of a number of arthropods at various cuticle temperatures.

and Beament (1945), to which they applied the terms 'transition point' and 'critical temperature'. These terms will again be adopted here, with the provision that they do not necessarily imply the acceptance either of the physico-chemical interpretation put on them in 1945 or of that proposed by Beament (1958*a*) for cockroach grease. The critical temperatures of these different species and stages in life history cover a wide range of temperature, from that of *Calliphora* to that of the argasid tick *Ornithodorus*, and if one compares them with values obtained by inspection of the published curves of earlier workers, either on whole animals or on isolated waxes, the correspondence is indeed close (Table 1). The permeabilities in Figs. 2–5 have not been reduced to a basis of unit surface area; for this reason the curves do not reveal the comparative permeabilities of different animals (see p. 418). This procedure has been adopted because of the great difficulty in estimating the true surface area of an insect. But where previous workers have not only used the same species (*Rhodnius*) as one involved in the present investigation, but have also adopted a formula (Wigglesworth, 1945) relating weight to surface area, it is justifiable to apply this formula to the new data and to compare the three investigations in the same terms.

Table 1. *A comparison of the transition temperatures* (° C.) *of the animals in the present study with those suggested by previous authors*

(V.B.W., Wigglesworth (1945); A.D.L., Lees (1947). Figures for isolated waxes from Beament (1945).)

Animal	Present study	Previous whole animal and source	Isolated wax
Pieris larva	38	35–45 V.B.W.	42–47
Pieris pupa	58	55–60 V.B.W.	60–65
Tenebrio larva	51	52 V.B.W.	50–55
Tenebrio pupa	48	52 V.B.W.	—
Ixodes adult female	33	30–35 A.D.L.	—
Ornithodorus adult	64	65 A.D.L.	—
Nematus larva	33	30–35 V.B.W.	30–35
Calliphora larva	38	35 V.B.W.	35–40
Calliphora pupa	51	50–55 V.B.W.	47–52
Rhodnius nymph	58	55–60 V.B.W.	55–60

In Fig. 6 we have the relationships for the *Rhodnius* nymph, taken from Wigglesworth (1945), Holdgate & Seal (1956) and from Fig. 4. The information is all shown according to the previous convention which makes no correction for the change of saturation deficiency with temperature, and since both the previous workers used *air* temperatures as the abscissa, curves for both air and cuticle temperatures have been included in the information extracted from Fig. 4. These curves emphasize more than ever the advantage of expressing results of this kind on linear scales and corrected for changes in saturation deficiency (Beament, 1958*a*); they also show that, despite the refinements adopted in the present method of investigation, the correspondence between the results now obtained and those of Wigglesworth (1945) are very close indeed—so much so that if Wigglesworth and Beament had adopted a suitable correction for saturation deficiency much of the more recent controversy over the existence of transition temperatures might never have occurred. But it is of some interest that the curve from Holdgate & Seal's (1956) paper shows a rate of evaporation which is consistently and at all temperatures two to three times greater than the rates represented by the other two curves, and that a discontinuity is much less obvious in their curve—indeed a transition point cannot be found with certainty even if their information is transformed to constant saturation deficiency and plotted on a linear scale. It is tempting to compare this curve with those of Fig. 1, both in smoothness and in rate of loss, and to suggest that the three curves are representative of insects which have suffered superficial damage.

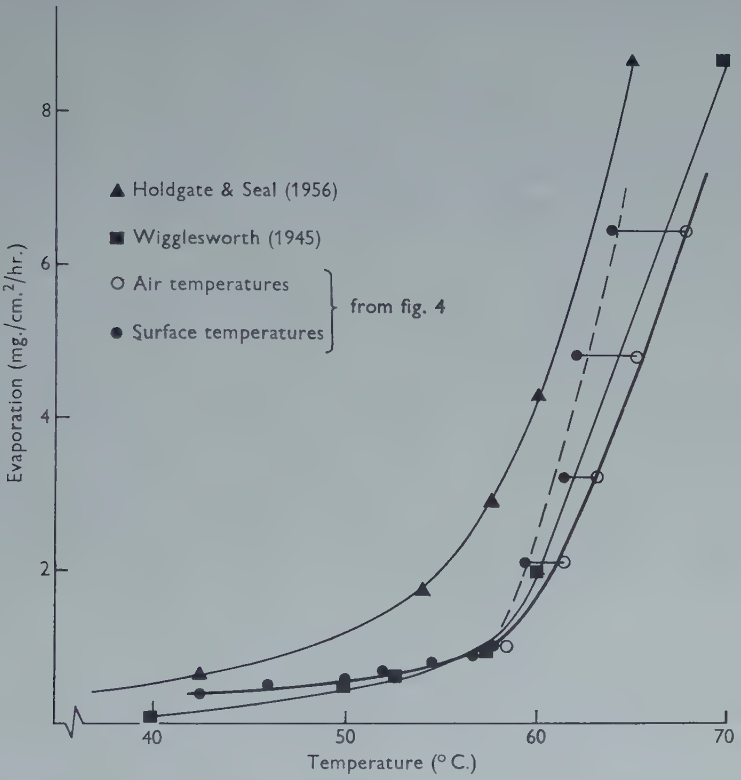


Fig. 6. Graph comparing the results of three investigations into the relationship between evaporation from a large nymph of *Rhodnius* in dry air and temperature. Values from Holdgate & Seal (1956) and Wigglesworth (1945) plotted at air temperatures. Data from the experiments giving the corresponding curve of Fig. 4 are plotted both at air, and at corresponding cuticle, temperatures.

Particular attention has been paid in these curves (Figs. 2–5) to the region of transition; below the critical point all these curves show a small and nearly linear change of permeability with temperature, corresponding to the state of affairs at lower temperatures in the cockroach. Admittedly there are few points on these lower regions of the curves, and if we are to apply the analysis which proved of value in investigating the waterproofing system of the cockroach they must be determined more critically. Above transition, in the cockroach, where it was assumed that the curve showed the property of completely disorientated grease, the curve was again linear with small slope. But in these new curves, so far as we have investigated higher temperatures, the curves continue to rise steeply from the point of transition. In these hard waxes then, we do not so far appear to be dealing with systems sufficiently similar to that of the cockroach for similar methods of analysis to prove fruitful at this juncture; hence we must investigate the circumstances on either side the discontinuity in various ways in order to get further information.

TEMPERATURE CYCLES

An observation by Wigglesworth (1945) forms the starting-point of the next series of experiments. He showed that if the larva of *Rhodnius* is exposed to a high temperature the impermeability of the cuticle is permanently impaired; this conclusion was based on alternate recordings of water loss at 80° and 50° C.—temperatures which he believed, and we have now confirmed, to be on either side of the transition point for this insect. The extent of the permanent damage was represented by a twofold to fourfold increase in water loss at the lower temperature, after exposure to the higher one. On the other hand exposure to temperatures above the critical point does not produce any permanent effect on the permeability of cockroach grease below the critical point (there may be some hysteresis or a slight shift in the actual critical temperature due to evaporation of solvent). The cockroach system is reversible with temperature and this is readily explained by the mobility of the grease molecules and their re-orientation against the wet surface of the epicuticle. To what extent does the behaviour of hard waxes differ?

Method

Where successive decrements of temperature are used in a series of determinations in the balance, great care must be taken to ensure that a dry atmosphere is maintained in the box. When the temperature of the equipment is raised, the enclosed air expands and some escapes; if the box is cooled, atmospheric air—which will have an appreciable humidity—will be drawn in. So, for experiments in which the temperature is lowered, a small but sufficient stream of dry air must be fed continuously through the box while it is cooling. With this particular equipment it was found that even if dry air at 20° C. was blown through the box at a rate of 3–4 l. per minute, when the control was set to maintain 40° C. the recording thermistor registered a drop of less than half a degree.

Results

Fig. 7 is a composite figure showing the effect of various temperature cycles on large nymphs of *Rhodnius*; each cycle shows the property of an individual, but the curves have been adjusted along the abscissa so that the vertical portions of the curves (*P*–*Q*) lie on one straight line—an adjustment involving shifts of less than 2° C. between the various curves. The sequence in which determinations in each cycle were made is indicated by arrow-heads. It is quite clear (e.g. curve *A*) that Wigglesworth's original observation has been confirmed, i.e. exposure to a temperature well above the transition point permanently impairs the waterproofing at lower temperatures. One must bear in mind that Wigglesworth quoted air temperatures, and that the extent to which the air temperature must rise in order to obtain values towards the upper end of the cuticle temperature line *P–Q* is much greater than the projection of *P–Q* on to the temperature axis. What is more important is the discovery that once the waterproofing system has been subjected to a temperature well above the transition point the sharp discontinuity also disappears permanently. Thus the heat-treated waterproofing system of *Rhodnius* has a permeability complexly related to temperature, and differs entirely in this respect from the cockroach. There is nothing particularly unusual about a permeability/temperature curve of shape *A*, but the existence of this relationship in heat-treated

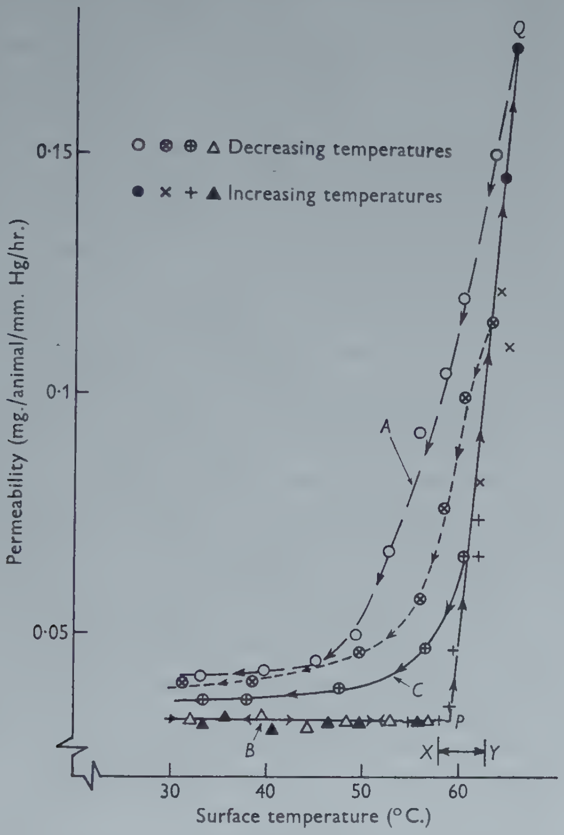


Fig. 7. Graph showing the permeability to water of the cuticle of *Rhodnius* nymphs during different temperature cycles. The information from four individuals has been adjusted along the temperature axis so that vertical portions of the curves lie on a line *PQ*. For further explanation, see text.

wax makes the permeability-temperature relationship of the natural cuticle seem all the more remarkable.

Now consider curve *B*; an untreated individual can be taken up to a temperature quite close to its transition point (subsequently determined) without causing any detectable change in permeability/temperature relationship at lower temperatures. This is in marked contrast with what happens at and above the transition temperature—for below that point the system is very temperature-stable. From curve *C* we learn that one has only to increase the temperature of the surface slightly above the transition point to get some permanent impairment of permeability and loss of sharpness of transition. All the curves which were obtained with decreasing temperatures (i.e. which start from the line *P–Q*), suggest that the property of that cuticle approaches the line *P–Q* asymptotically. This is confirmed by experiments in which nymphs, exposed to temperatures above transition, have been cooled to room temperature and then measured with a series of increasing temperatures.

The general conclusion from these experiments is that they confirm the abruptness of the start of transition; they show too that the changes above the commencement of transition may be progressive with further increase in temperature until the maximum permanent impairment of waterproofing has been reached (though of course the actual temperature difference between points *P* and *Q* is small). From Fig. 7 it is certainly fair to limit the extent of the entire transition phenomenon to the interval of surface temperature indicated by *X–Y*—three or four degrees at most. Results similar to these have been obtained using both the larva and pupa of *Tenebrio* and the pupa of *Pieris*; it may be noted that although we have not demonstrated (Fig. 5) a sharp discontinuity in the curve for the locust hopper, nevertheless, exposure to temperatures along the steep upper portion of its permeability/temperature curve does lead to a permanent increase in permeability at lower temperatures, and to a less steep curve. This discovery serves to illustrate an important proposition. If we find a sharp discontinuity in a permeability/temperature curve we can call this a transition point, and it is simplest to assume that all transition points are expressions of the same type of physico-chemical change (except in the cockroach for which the curve is markedly different). But suppose we do not find a sharp discontinuity, but merely a change in slope over a particular temperature interval. If we can further demonstrate a permanent increase in permeability after exposure to a higher temperature and a marked smoothing out of the curve, we may at least assume that something similar to a transition phenomenon is taking place over that range of temperature. This argument becomes of greater importance in the experiments next reported.

PERMEABILITY AT LOWER TEMPERATURES

In the curves of Figs. 2–5 particular attention was paid to determining the transition regions, and comparatively few readings occur in the regions of lower temperature. Straight lines have been drawn through those points to construct the curves, but if we are to attempt a determination of the form and slope of the lines for physical analysis the points must be determined with greater accuracy. The order of water loss of some of these insects at room temperatures is remarkably low; even with such sensitive apparatus as we are now using, it may be difficult to detect a sensible amount of water loss for an interval of less than 10 min.; and, surprisingly, readings may have to be extended over much more than half an hour to obtain that necessary agreement between successive values which indicates that a steady rate of evaporation has been established (see p. 406). But when one also takes account of the need to construct curves from determinations all made on one individual the complete series of measurements may extend over 3 or more days. The validity of the information so obtained rests on the assumption that the properties of the waterproofing system remain stable for this length of time after the animal has been killed (it is shown on p. 405 below that this assumption is justified). But as one of the basic measurements we are making is of a change in weight, we must guard against changes other than water loss—in particular, oxidation and bacterial decomposition—which might alter the weight of the specimen. For this reason air was replaced with nitrogen during the long-term experiments. Some of the short-term experiments were repeated with nitrogen, as a check, but this made no difference to the results obtained.

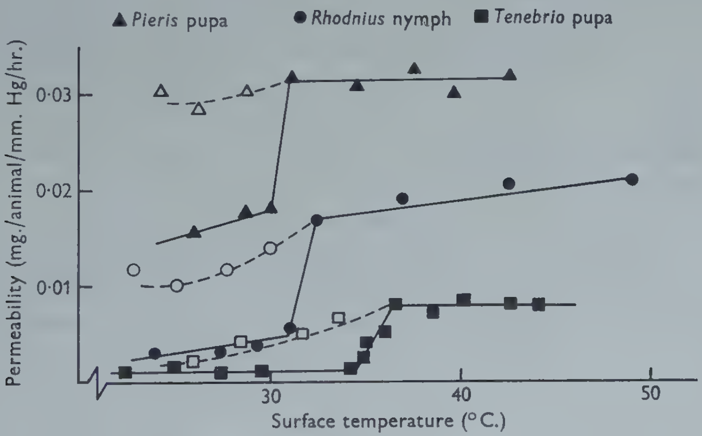


Fig. 8. Graph showing the permeability to water of three different insects at lower temperatures: *Rhodnius* nymph 1 month after moulting, *Tenebrio* and *Pieris* pupae each 5 days after moulting. The broken curves show permeabilities determined after each individual has been exposed to the highest temperature represented on the corresponding continuous line.

Results

In Fig. 8 we show the permeability of three insects at lower temperatures: the pupae of *Pieris* and of *Tenebrio*, each killed at 5 days after pupating, and of a *Rhodnius* nymph 1 month after moulting. The broken line of each curve shows the permeability redetermined after the individual has been exposed to the highest temperature for which a point of determination appears on the associated unbroken curve. None of the specimens has been allowed to approach the temperature of transition as recorded in Figs. 3 and 4. Now each of these curves shows a discontinuity of a kind not dissimilar to a transition phenomenon; the discontinuities are not particularly sharp, but it must be remembered that the actual changes in permeability which take place across the discontinuity are very small and that they might appear much more sharp if they were followed by a long, almost vertical excursion of the curve. The suggestion of a transition phenomenon is supported by the demonstration of irreversible increase produced by exposure to higher temperature: except in respect of this irreversibility each curve closely resembles the permeability/temperature curve for the cockroach (Beament, 1958*a*) in which the curve is abruptly displaced upwards at the transition point.

On the other hand, when the lower temperature regions of the curves of the larvae of *Calliphora* and *Pieris* and of the adult *Ixodes* were investigated in detail, no indication of a second smaller discontinuity was found; the significance of these differences will be discussed later (p. 416). But where two breaks clearly occur in a curve an important issue arises. In the cockroach we visualized the transition to be the disorientation of a monomolecular layer of grease. But it is extremely difficult to visualize a monolayer with two stages of transition, especially so, for example, in *Rhodnius* where the two breaks occur some 25° C. apart and do not seem to be related in any way—disorganizing one has no effect on the other. There is enough wax on these insects (Beament, 1945) to provide for twenty-five monolayers. However, if we suggest two *independent* monolayers, the difference in their properties indicates that they must be composed of waxes having very different average molecular lengths.

Before examining this possibility, it is interesting to consider the absence of any indication of a second transition phenomenon in the results of previous workers. Though the permeability of a cuticle remains sensibly constant over a lower range of temperature (i.e. the curve as plotted on the axes chosen in this paper is sensibly parallel to the abscissa) the measured rates of evaporation over this region are rising exponentially with temperature, keeping pace with the increasing saturation deficiency of dry air. When transition occurs at a high temperature, the change in evaporation rate over a small interval of temperature is great, not only because of the change in permeability, but because it is being measured in high saturation deficiencies; smaller changes in permeability associated with lower transition points have to be measured in lower saturation deficiencies, with the result that simple apparatus cannot detect them.

TRANSITION POINT AND THE AGE OF THE ANIMAL

Wigglesworth (1945) pointed out that a constant rate of water loss was typical of each species and of each stage of the development of the species; Beament (1946, 1951) observed a similar phenomenon in the waterproofing waxes of egg-shells. On the other hand, Holdgate & Seal (1956) demonstrated a substantial difference in the water loss/temperature curves as between young pupae and older pupae. Briefly, they found that a newly moulted animal is more permeable to water than an older one at all temperatures, and that if both curves are plotted on the same axis, the curve for the younger animal starts to rise steeply at a lower temperature than that for the older one. Holdgate & Seal drew attention to a waxy bloom on the outer surface of the cement covering the cuticle of *Tenebrio*, and they associated the differences in the curves they obtained with the secretion of the cement layer. It is of special interest to note their statement that the wax exposed on the surface of the newly moulted *Tenebrio* pupa melts at 40° C., whereas the waxy bloom appearing above the cement melts in the region of 60–80° C.

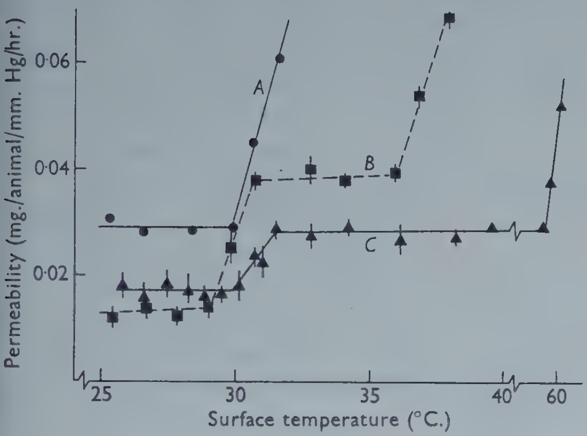


Fig. 9

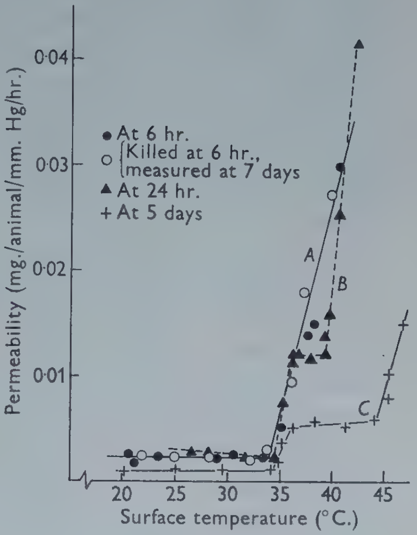


Fig. 10

Fig. 9. Graph showing changes in the permeability/temperature relationship of pupae of *Pieris* with age. *A*, pupa killed 6 hr. after pupation; *B*, killed 24 hr. after pupation; *C*, killed 5 days after pupation. Vertical lines through individual values indicate the maximum limits of experimental error in the determination.

Fig. 10. Graph showing changes in the permeability/temperature relationship of *Tenebrio* pupae with age. *A*, pupae killed at 6 hr. after pupation and measured either then or 7 days later; *B*, pupae killed and measured at 24 hr. after pupation; *C*, pupae killed and measured at 5 days after pupation.

For investigation of the ageing of the waterproofing system from the time of ecdysis, the pupa of *Tenebrio* and the pupa of *Pieris* were selected. In Fig. 9, curve *A* shows the permeability/temperature relationship of a *Pieris* pupa killed with hydrogen sulphide 6 hr. after moulting; there is one sharp transition point at about 30° C. and above it the curve rises steeply for so far as it has been determined. This animal has only its primary wax layer. When, however, a pupa is killed at 24 hr. (Fig. 9, curve *B*) there are two discontinuities, at 30° C. and again at 36° C. When a pupa is killed at 5 days (curve *C*) one transition occurs at 30° C. as before but the other is not found till the skin temperature reaches 59° C. Curves *B* and *C* have been determined with greatly extended periods of desiccation, and the limits of accuracy of the individual points have been added, to make clear that the distinction between the two discontinuities is real and to emphasize the sharpness of the lower discontinuity. One should note that it is the upper point of curve *C* (transition at about 60° C.) which the earlier workers referred to as 'the' transition temperature of the *Pieris* pupa.

Similar experiments of pupae of *Tenebrio* are illustrated in Fig. 10. Making due allowance for differences in technique and for the scales on which the results are expressed, there is no great discrepancy between the data of Holdgate & Seal (1956) and the results of this experiment. Certainly, the characteristics of waterproofing change appreciably with the age of the living pupa. A transition point at 35° C. remains relatively constant in position throughout the life of the instar. A second transition point (curve *B*) appears at about the time of secretion of the cement; at that time it is not much higher in temperature than the first transition point but there is no doubt (on grounds of experimental error) that there are two separate breaks in curve *B*. As the pupa ages, the temperature of the second transition point appears progressively higher (curve *C*). The accuracy of this experiment is such as to indicate clearly that there are still only two transition points, as distinct from a series of transition points added at successively higher temperatures with age.

As in some of the previous experiments, the measurements on a particular specimen used to provide the data in Fig. 10 often extended over 3 or 4 days. It is very important, therefore, to note that curve *A* is drawn through two series of determinations; one set of points has been derived from a pupa immediately after it had been killed, while the other comes from a pupa killed at an identical age but stored for 7 days at 15° C. before the measurements were made. There is no sensible difference between the curves representing the trend of the two series of points. This information considerably enhances the value of the extended experiment, for it would seem to indicate that the properties of the waterproofing system remain very stable for some time after death even though they may be changing in the living animal. Other implications of this experiment are discussed below (p. 416).

THE DISTRIBUTION OF CUTICULAR WATER DURING EVAPORATION

Beament (1948) points out that unless measurements are made under conditions of a steady rate of water loss, the results obtained from experiments on transpiration from cuticle and from models of cuticle could be most misleading; and in the present work emphasis has been laid on the need to obtain consecutive readings of close agreement at any one closely controlled temperature before adopting a measurement of permeability. But the changes which occur prior to the establishment of a constant rate of flow through the cuticle at any one temperature are not without interest, for we can deduce some information about the distribution of water in the outer cuticle from them.

Fig. 11 has been constructed from data obtained from a *Tenebrio* pupa shortly after the secretion of the cement. The permeability/temperature curve of this specimen has been drawn, but for clarity only a selection of the points which determine it has been given. In addition, there are several points which have been obtained by calculating the apparent permeability of the cuticle from the rate of water loss which is measured immediately after the apparatus has taken up a new temperature. The readings shown in the figure were obtained in alphabetical order. Consider, first, circumstances below the lower transition temperature. At about 27° C. the permeability measured when the rate of water loss is constant is indicated by *b*. The temperature is raised, and as soon as it becomes stable the rate of water loss first measured gives us a permeability *c*; it is significantly higher than the steady rate (*d*) which obtains some 30 min. later. This phenomenon is observed with each temperature increment over the first portion of the permeability/temperature curve. Thus although the true permeability of the cuticle is not altering sensibly in this temperature range, each time the temperature is raised the specimen loses some water in addition to that which can be accounted for by its permeability. In contrast, if we lower the temperature (*f* to *g*) the initial rate of evaporation, and subsequent values at the new lower temperature, agree closely; judged by the line of the graph the permeability (*g*) is low, but the departure is hardly significant.

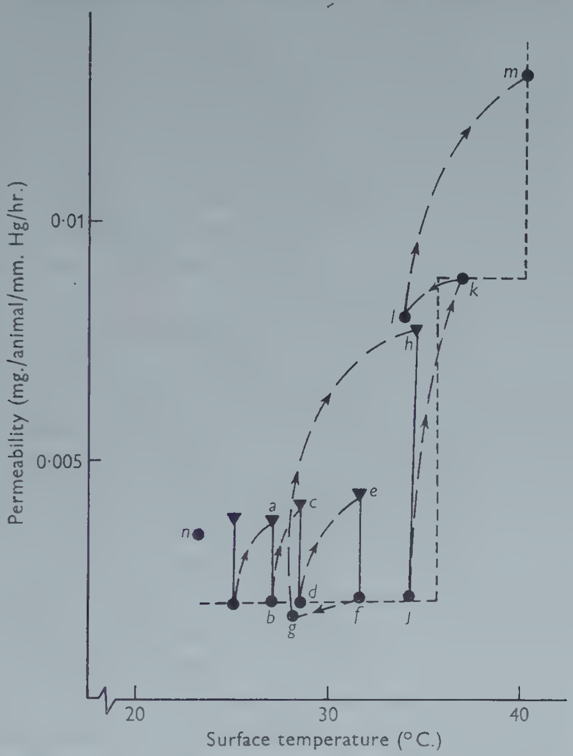


Fig. 11. The permeability/temperature relationship for a *Tenebrio* pupa at 24 hr. old. Certain experimental values have been added: *a, c, e, h* are apparent permeabilities immediately after a change in temperature; *b, d, f, j*, are corresponding true values; *g, k, l, m, n*, are true values attained immediately after a change in temperature. Determinations were obtained in alphabetical order (symbol *i* not used). For further explanation, see text.

We see, too, that the larger the increment (*g* to *hj*) the greater is the initial rate of water loss (giving *h*) though the steady value (*j*) comes closely on to the line through *b*, *d*, *g* and *f*. Interesting as it may be to pursue this kind of experiment in greater detail, a temperature increment cannot be made in a few seconds (it does take only a few minutes) and the cuticle obviously starts to react to an external change in temperature and saturation deficiency immediately the temperature of the air surrounding it starts to change.

Now consider events at temperatures above the lower transition point. As soon as a new temperature has been obtained the rate of water loss seems to settle to a constant value at once (*k*); it does not take longer to adjust the temperature in this range than it did at lower temperatures. If the specimen is now cooled to a temperature lower than the lower transition point (*l*) we still get steady rates of evaporation simultaneously with the establishment of the new temperature; the actual permeability is of course now permanently impaired, and the proximity of points *l* and *h* is coincidental. The picture of events is completed by taking the specimen well above its higher transition point (*m*) and then returning it to the lowest temperature of the whole experiment (*n*). In both cases steady readings are obtained as soon as the new temperatures have been established.

We next examine in a similar fashion the experimental data from which curve *A* of Fig. 10 was obtained; here we have the freshly emerged pupa of *Tenebrio* with only the primary wax layer on its surface. We find that throughout the experiment the rates of water loss are sensibly constant at each temperature, from the time of the establishment of the new temperature. The same remarks apply to the experimental records for the *Calliphora* larva and for the newly formed pupa of *Pieris*, but variations similar in nature to those illustrated in Fig. 11 occur in the experimental records for the mature *Pieris* pupa and for the mature *Rhodnius* nymph.

These findings must now be considered in the light of two theories. Beament (1954) suggested that some of the unusual properties of insect cuticle, particularly its ability to obtain water from subsaturated atmospheres and its asymmetric behaviour when the direction of flow through it was reversed, might be more readily understood if one considered the distribution of water across the cuticle during conditions of steady flow. Across each component of a cuticle transmitting water there must be a concentration gradient, falling in the direction of flow, and the greater the rate of flow the steeper the gradient (Fig. 12*A*). This picture supposes an extremely simple structure for cuticle, comprising one uniform hydrophilic phase and a single layer of wax outside it. The gradients through the hydrophilic layer have been greatly exaggerated for clarity, and no account has been taken of the comparative resistance to water flow of the two components comprising the cuticle model. In fact, the wax is so much more resistant that, so long as we consider only low rates of flow entirely in the outward direction, the change in concentration at the wax-hydrophilic cuticle interface is negligible compared with a corresponding change in saturation deficiency at the outer surface of the wax (Fig. 12*B*). We may regard the hydrophilic cuticle as a reservoir of water. Our experiments with animals (*Calliphora* larvae and young pupae of *Tenebrio* and

Pieris) lend support to this conclusion: we believe these animals to have a hydrophilic cuticle covered only with a primary wax layer, as in this simple model. When the external saturation deficiency changes, the rate of flow changes immediately, and proportionally to it. Indeed we assume in our calculation of the permeability of the cuticle (meaning of the wax layer) that the change in gradient across the wax is the change in saturation deficiency.

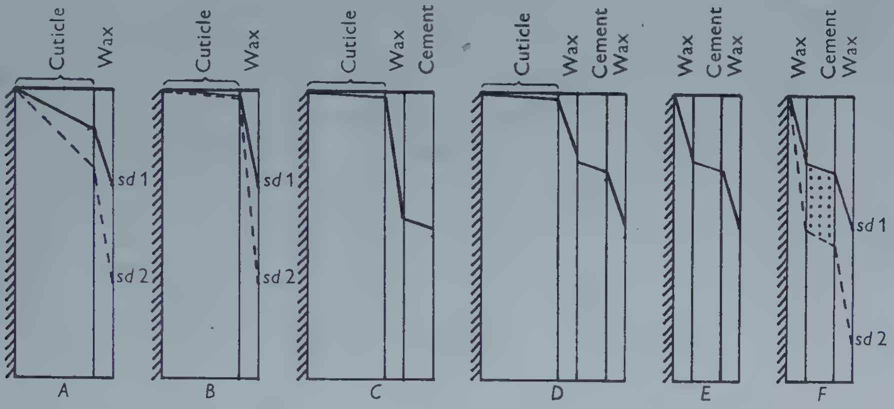


Fig. 12. Representations of sections of cuticle through which water is transpiring from the inside of an animal (left of each diagram) into dry air (right of each diagram). Diagonals through sections indicate the form of the gradient of water content causing transpiration. *sd* 1 and *sd* 2 indicate two external saturation deficiencies, of which *sd* 2 is twice *sd* 1. In section *F* the stippled area represents the amount of water which the cement must lose during the change from conditions of steady flow at *sd* 1 to similar conditions at *sd* 2. The relative thicknesses of the components of the cuticles are not to scale. For further explanation, see text.

But are we correct in assuming that the cuticle of an older pupa can be represented by this simple model? We have (p. 404 above) questioned the possibility that one wax layer could show two transition points and have suggested that two such discontinuities in the permeability/temperature curve of a single monolayer would be even less readily accepted. The upper transition temperature appears only after secretion of the cement, and to represent the cement a layer which must certainly be more hydrophilic than wax must be added to the model (Fig. 12*C*). If the wax is still the real barrier to the flow of water we must still suppose that almost all the gradient causing flow must be across it; and we would expect the system to respond rapidly to changes in external conditions, for they will produce only small changes of gradient in the cement, as in the hydrophilic cuticle.

But if we assume that there is a second monolayer of wax on the outer surface of the cement (Fig. 12*D*) (not the waxy bloom of Holdgate & Seal (1956), though this may well arise from such a monolayer) many more of our observations can be explained. We can now suggest that the two transition points belong respectively to the inner and outer wax layers; this correlates exactly with our experiments on animals of different ages within the instar. We can continue to regard the main inner hydrophilic cuticle as a reservoir of water and simplify our model of gradients to Fig. 12*E*. The resistance to flow now occurs in two wax layers and the gradients across them will be steep. When the rate of flow increases, the gradient across the cement may not change greatly in accommodating that greater flow, but the distribution of steeper gradients across the wax will cause a substantial alteration in the concentration level within the cement (Fig. 12*F*). In other words, when the rate of flow is increased the cement must lose some water before it can establish its new slight gradient.

If we now consider Fig. 11, we can see that all stages of this argument fit with our findings. (This figure shows *permeabilities*; the rate of flow through the cuticle increases with temperature, proportionally with the increase in saturation deficiency, when permeability remains unchanged.) Each time the temperature is raised and *until* new stable conditions obtain, the actual water of evaporation which is measured can be considered as two fractions. One represents an increased saturation deficiency; the other represents a reduction in the water content of the cement. The cement is thin and its water content at saturation will not be great; if water could escape from it freely the water content of the cement would quickly reach its new value. But if the surface of the cement has a highly impermeable wax barrier on it a considerable period of time must elapse after a temperature increment before the initial rate of water loss comes down to a steady value. The greater the change in rate of flow, the greater is the alteration of the water content of the cement, as is seen by comparing *d*, *e*, *f* with *g*, *h*, *j* in Fig. 11. After a decrease in temperature the rate of water loss should be low during the period of transition while the cement is accumulating water. This is not convincingly shown; experimentally, it takes much longer to obtain a particular decrement by cooling than a corresponding increment of temperature by heating, and we may not have been able to obtain the vital reading (but see below and Fig. 13).

But these theories are of greater interest still when we consider the events above the lower transition temperature in Fig. 11. If the primary wax layer of the pupa retains its properties of permeability after the secretion of the cement, it should now be very permeable to water. Water can flow from the reservoir of the hydrophilic cuticle into the cement. If the remaining barrier with high transition temperature is on the outer surface of the cement, the system is now represented by Fig. 12*B*, the outer wax layer representing wax, and the cement representing cuticle. We should expect, and we indeed find, that changes in the rate of flow can be accommodated very rapidly.

THE PUPARIUM MODEL

The transient conditions we have analysed in the preceding section are revealed in changes of very small magnitude. The actual amounts of water which might be lost by the cement over any one change of rate of flow are extremely small, though calculation shows their order to be reasonable. Much more satisfactory confirmation of our ideas would be given by a system, similar in essentials to the wax-cement-wax sandwich we have postulated, but in which the changes to be observed were on a greater scale. Fortunately, one such model is readily available. During the development of *Calliphora* there is a stage where the puparium surrounds the pupa. Neither cuticle, so far as we know (Wolfe, 1954), has any cement, and thus the waterproofing system consists of two primary wax layers with a great thickness of puparial cuticle between them. There is also an air gap between the puparial cuticle and the pupal wax but its presence does not affect our arguments. The principal difference between this waterproofing system and the one we have postulated for the older pupa of *Tenebrio* is that the outer wax (on the puparium) has the lower transition temperature. Not only can we investigate this system by taking the intact animal through a wide range of temperature, but (see Wigglesworth, 1945) we can cut away a large part of the puparial cuticle without damaging the pupa and investigate the properties of the inner layer alone.

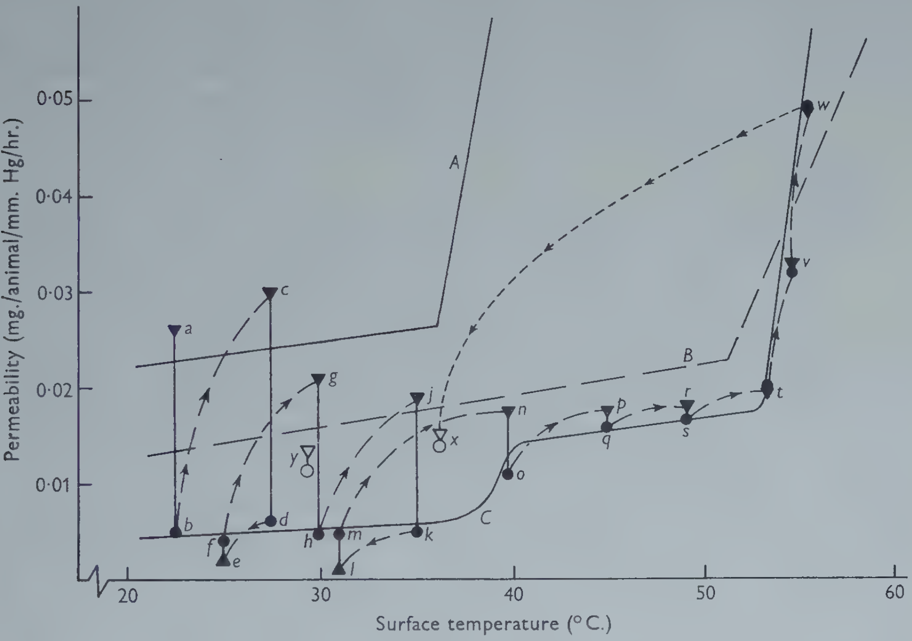


Fig. 13. Permeability/temperature curves for *Calliphora*. *A*, puparium at 24 hr., having only the larval wax layer. *B*, the pupa alone at 3 days old (obtained by dissection). *C*, the intact puparium containing a pupa at 3 days. Experimental values have been added to curve *C*; triangles indicate apparent permeabilities immediately after a change in temperature, and circles the corresponding true permeability. Information has been obtained in alphabetical order (symbols *i* and *u* not used).

The results of these experiments (Fig. 13) entirely substantiate our theory based on events in the cuticle of the older *Tenebrio* pupa (Fig. 11). When only one wax layer is present, either naturally or effectively, above the transition point of the puparial wax (or artificially, after dissection of the puparium) a steady rate of water loss is rapidly established at each new temperature. When two impermeable barriers enclose the thick cuticle of the puparium, new rates of flow are accommodated only very slowly, and the apparent values of permeability calculated immediately after new conditions of temperature have been established depend on the direction in which the temperature has changed.

THE ACTION OF ADSORPTIVE DUST

We have not been able to obtain *Tenebrio* pupae in which the cement has been produced and which do not show two transition points; indeed we do not know whether there is a natural stage in development at which the pupa has cement but is without the wax we have supposed to constitute an impermeable barrier above it. But since we have postulated this outer wax layer, it would be desirable to supplement the information of Figs. 11 and 13 by carrying out experiments after we have tried to remove that wax. Holdgate & Seal (1956) measured the water loss from *Tenebrio* pupae from which they had washed the waxy superficial bloom with chloroform. However rapidly washing is carried out, contamination of the cuticle with polar solvents (Beament, 1954) may produce far-reaching effects which are by no means understood. It would be unwise to draw conclusions from these experiments.

In the earliest experiments of this paper (p. 394) we showed that however gently an insect is handled its permeability is increased. We have actually observed this with a number of different species in the course of the work, but in reviewing those very qualitative results it is rather surprising to find that it is the insects *with* a cement layer which are most obviously changed by handling. It may be worth noting a previously unpublished observation that the cuticle of *Periplaneta* is apparently unaffected by gentle handling—and certainly this has no effect on the appearance of a sharp transition point—whereas handling destroys the sharpness of the transition phenomenon in some other insects; and it is particularly worth contrasting the effect of handling the new, and the mature, pupa of *Pieris* (Fig. 14). These experiments are qualitative but they clearly suggest (*a*) that the proportional increase in evaporation produced by handling an older pupa (with cement) is much greater than that produced by handling a young pupa, and (*b*) that handling an older pupa interferes with the abruptness of the upper transition point alone.

The second observation strengthens our hypothesis that the upper transition phenomenon is associated with the outer surface of the cement. The first is much more surprising; if the cement protects the primary wax layer, why should the permeability of the cuticle be increased so greatly by interference with the upper surface alone? It is clearly necessary to obtain quantitative information about this; and the experiments of Fig. 12 suggest a way in which adsorptive dusts can be used.

The mode of action of the so-called 'inert' dusts, which cause increased transpiration, has been a source of controversy for many years (see, for example, Beament, 1956). Much of the argument has been about the precise mechanism by which the dust works—whether it adsorbs wax or whether it abrades and damages the wax mechanically. But such hypotheses necessarily depend on the structure of the cuticle; they have not taken into account the possibility that there could be a further waterproofing layer on the outside of the cement. Suppose we could provide circumstances in which a dust can act only by adsorption when applied to the surface of a mature pupa; we should get a specimen showing only the characteristics of a young pupa (except that its primary wax layer will be covered by cement alone). We can, additionally, apply such a dust to a newly emerged pupa and find its effect on a system with a single wax layer. If the experiment proves successful, we shall also throw considerable light on the long-standing problem of the mode of action of dusts.

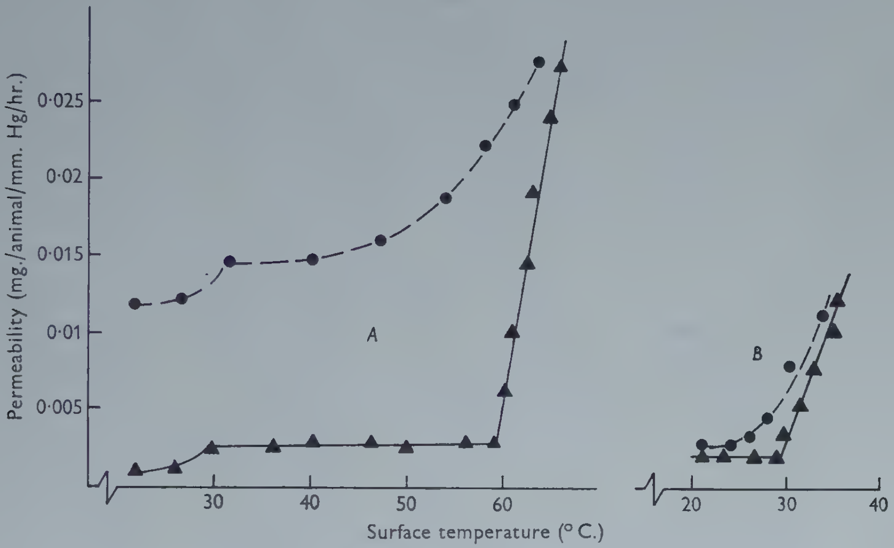


Fig. 14. The increase in permeability produced by handling pupae of *Pieris*. *A*, pupa 5 days old; *B*, pupa 12 hr. old.

Animal charcoal ('activated' charcoal) was selected for these experiments; not only has it excellent adsorptive properties but it is also very soft indeed. The sample used in the experiments reported below came from the same jar as that used by Holdgate & Seal (1956). These authors rolled pupae in this dust for a stated number of times and showed an increase in evaporation correlated with the amount of rolling. We have not attempted to copy this technique exactly; on the other hand, every effort has been made to provide conditions in which the dust could adsorb without abrading.

The specimen was placed on a rubber bung at the bottom of a 3 cm. diameter glass tube, 1 m. long. This tube was mounted vertically; into its upper end was inserted a short glass tube of considerably smaller diameter which formed one limb of an inverted U of about 60° included angle. The other limb of this U-tube—about 1 m. long—thus rose at an angle from the bench. Its lower end was led into a vessel containing a sample of animal charcoal and a glass nozzle on a flexible sleeve. By blowing compressed air through the nozzle a cloud of charcoal particles could be blown up the inclined tube; heavier particles were precipitated and ran back to the vessel. By adjusting the rate of air flow an alutrate of very fine charcoal dust was sent into the top of the vertical tube containing the specimen and settled on it. When the pupa was uniformly covered in a dense black film the bung supporting it was carefully extracted. As much superficial dust as possible was removed from the pupa by playing a fine jet of compressed air against its surface and it was then transferred to the balance; its surface was still a dull grey colour. Some animals were successfully placed on the balance merely by tipping the bung; others were manipulated by means of a minute suction tube which made contact with only a very small area of the surface.

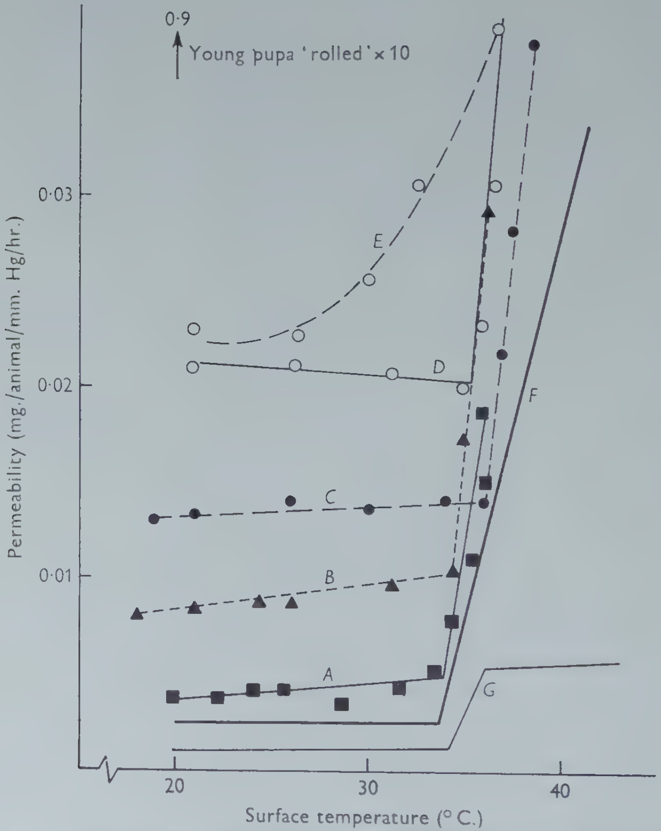


Fig. 15. Permeability/temperature curves for pupae of *Tenebrio* after various treatments with animal charcoal. *A*, pupa 12 hr. old, having a surface covered with dust (adsorption alone); *B*, pupa 5 days old, having a surface covered with dust (adsorption alone); *C*, pupa 5 days old, rolled once in dust; *D*, pupa 5 days old, rolled ten times in dust; *E*, the pupa of curve *D* measured after heating to the highest temperature indicated on curve *D*; *F*, an intact pupa 12 hr. old; *G*, an intact pupa 5 days old.

For comparative purposes other pupae were placed in the long wide tube and about a cubic centimetre of animal charcoal was added. The tube was closed at both ends with bungs and rotated very slowly so that the specimen slid gently from one end to the other along the inner surface of the glass and rolled in the dust at either end.

The permeability/temperature curves of these charcoal-treated animals are shown in Fig. 15. Apart from the curves on that figure, attention is drawn to the exceptionally high permeability of a freshly emerged pupa which had been treated by rolling it in dust for ten revolutions of the tube; the animal lost water so rapidly that one could not use present techniques to construct a permeability/temperature curve for it. Exposing the primary wax to a soft adsorptive dust with movement must lead to heavy damage. By contrast (Fig. 15, curve *A*), if a freshly emerged pupa is coated in an alutrate of charcoal but movement is avoided as far as possible, there is very little increase in permeability.

When mature pupae are treated with dust their permeability/temperature curves show three features, each of considerable significance. Where an animal has been exposed—so far as this is experimentally possible—only to adsorptive forces all sign of an upper transition point disappears (curve *B*); not unnaturally, there are no upper transition discontinuities in the curves from animals which have also been rolled in dust (curves *C* and *D*). But the lower transition point is quite clearly defined in all these curves, whether the animals have been subject to adsorptive forces alone or whether they have been rolled in dust as well. Of course, when the animal is rolled in dust some gross mechanical damage must occur, and the more the pupae are exposed to this treatment the less abrupt is the lower transition discontinuity; yet even in the curve (*D*) obtained from the most damaged cuticle one cannot mistake the transition region, and from the arguments above (p. 400) our confidence in its reality is increased by taking such a specimen through a temperature cycle (curve *E*), when we obtain a smooth curve typical of permanently impaired waterproofing.

In addition, the experimental records show that every one of the animals represented in Fig. 15 attains a steady rate of water loss almost simultaneously with the establishment of new conditions of temperature. This evidence fits excellently with our suggestion above, namely, that we are here dealing with systems containing only one waterproof barrier.

Thirdly, there remains perhaps the most puzzling feature of Fig. 15, which confirms the suggestion originally made in connexion with the handling of pupae of *Pieris*. The charcoal-treated mature cuticle shows greatly increased permeability at all temperatures, even though it retains the characteristic lower transition discontinuity so sharply that in *this* respect we might suggest that the primary wax layer is entirely undamaged. When the insect is taken through a temperature cycle (curves *D* and *E*) the relation between the two half cycles is typical, but the entire phenomenon has been translated up the ordinate. This discovery, which is discussed below, may prove of significance.

DISCUSSION

Excepting the locust hopper, we have found at least one sharply defined discontinuity in each of the permeability/temperature curves of a range of different insect and acarine species. The nature of the discontinuity closely resembles that which Wigglesworth (1945) and Beament (1945) supposed to exist in experiments which technically were incapable of revealing a discontinuity precisely; wherever we can compare similar species the temperatures at which some of these breaks occur also correspond closely with the temperatures originally suggested by those authors and by Lees (1947). There is no doubt that the phenomenon we now describe is the one originally called a 'transition' occurring at a 'critical temperature'. We must make it clear that there is no single experiment in the paper by Holdgate & Seal (1956) which conflicts directly with the findings of a corresponding investigation in the present work; all discrepancies can be explained by differences in technique and in the parameters and scales chosen to display and examine results. Holdgate & Seal were quite justified in concluding that they could not show the existence of transition phenomena, and they were also probably correct in suggesting that changes in the permeability of wax layers on insects could not be explained by Müllerian transitions. It is unfortunate that they attempted a mathematical treatment of data all too scanty for the purpose, but not nearly as unfortunate as the suggestion by reviewers, such as Edney (1957), that the transition phenomenon is an artefact.

The whole of the evidence we now have is consistent with the following hypothesis: if two transition points occur, the lower is associated with the primary wax layer and the upper is associated with the outer surface of the cement. The critical temperatures suggested by earlier workers must be compared in some species with the upper critical point, and in others with the single critical point we have found. Wigglesworth (1945) and Beament (1945) thought that all transition phenomena were properties of the primary wax layer and we must now attempt to associate the behaviour of the cuticle with what we know of its structure. *Calliphora* larvae, puparia, and pupae (Wolfe, 1954) and *Ixodes* (Lees, 1947) have no cement; they have only one transition point. *Rhodnius* nymphs (Wigglesworth, 1947), *Tenebrio* pupae (Holdgate & Seal, 1956) and *Pieris* pupae have cement; they have two transition points. We know nothing of the nature of the cuticle surface in larvae of *Tenebrio*, *Pieris* or *Nematus*, though there are reasons (see for example, Way, 1950) for believing that they might have a cement covering. Malek (1958) proposes that the cuticle of the locust is very similar to that of the cockroach; it has a cement (see Kramer & Wigglesworth, 1950) but Beament (1955, 1958*a*) suggests that the cement on the cockroach is a discontinuous structure, so that *functionally* the grease is in a single layer over the surface. If the locust is like this it also would have one functional wax layer. Finally, *Ornithodorus* (Lees, 1947) has a cement layer; it is difficult to decide whether its curve (Fig. 3) shows a small break around 35° C. With such a scanty knowledge of cuticles, and until we have information on a very large number of different species and orders, we cannot make useful suggestions about types of waterproofing systems. The material question is: if a cuticle has a cement layer, but reveals only one transition point, does this represent the property of the primary wax layer, or of a wax on the cement, or can there be two layers with identical properties? In the three insects we have examined in most detail, two critical temperatures are associated with wax on either side of the cement; those two wax layers must have very different properties, the most likely basis for which must be a difference in mean molecular weight.

Consider the *Tenebrio* pupa. A primary wax with transition around 35° C. suggests a relatively low molecular weight, and the X-ray studies of Holdgate & Seal (1956) indicate that this wax melts at about 40° C., i.e. a few degrees above its critical temperature, which is precisely the relationship first suggested by Beament in 1945. Holdgate & Seal give 60–80° C. as the melting-point of the waxy bloom over the cement, a temperature bearing a similar relationship to the transition point of about 55° C. which we now associate with the surface of the cement. We must be careful to distinguish between a bloom—which is a discontinuous formation—and the imperforate layer which we think more likely to give great impermeability and sudden transition; but there is no reason why the bloom should not be associated with a continuous layer of wax at the actual surface of the cement. Whatever the detailed structure there is on the cement a wax of high melting-point, and therefore of high molecular weight; and its origin must be established. The cement is likely to be shellac (Beament, 1955), and although commercially obtained shellac includes waxes which may well be waterproofing materials there is no evidence in Wigglesworth's studies (1947, 1948) that the epidermal glands contain or secrete any wax. Holdgate & Seal show that the wetting properties of the cement surface change continuously from the time of its secretion, corresponding with the gradual appearance of the bloom. All this suggests that there is no wax in the cement when it is first secreted, but that wax subsequently migrates into it and through it. The most obvious origin of that wax is the primary wax layer. We suggest that the primary wax of *Tenebrio* is a solution of the hard outer wax in a volatile solvent and that it migrates through the cement to form the outer layer by the evaporation of volatile components there. The transition point of its primary wax may appear constant over a period of a week, but this does not disprove the existence of a solvent; the grease of the cockroach, which is known to contain volatile solvents (Beament, 1955), shows a change of only 2–3° C. when exposed on the living animal for a month (Beament, 1958*a*). Cockroach grease may change to a wax melting at 55–60 °C. on heating but such a change is only accomplished at room temperatures in about 6 months. Further comparison of the properties of the two wax layers on some of these insects with the grease of the cockroach supports this theory. *Tenebrio* pupae have transition points at 35° and 55° C.; *Rhodnius* nymphs at 30° and 58° C.; *Pieris* pupae at 30° and 60° C.; *Tenebrio* waxes melt at 40° and at 60–80° C.; cockroach grease changes from transition at 30° C. to a melting-point at 55–60° C. And further, the form of a wax bloom is characteristic of a process of crystallization by slow evaporation of a solvent.

Superficially, some of the evidence of Beament (1945) is against this theory. Extracted waxes on membranes and membranes of cast skins showed characteristics which we now associate with the outer wax layer, but the bulk of the wax extracted from the cuticle must have come from the *inner* primary wax layer. We do not have information species for species; but the melting-points quoted by Beament (1945) (e.g. 60° C. for the total extract of *Rhodnius* wax) cannot always be reconciled with Holdgate & Seal's figures (e.g. 40° C. for the melting-point of

Tenebrio primary wax), or with the low transition temperatures of the main primary layer in many of the insects studied in this paper. But the original records show that Beament (1945) extracted waxes from cast skins which had been collected over periods of months if not years, and some of the cast skins he used as membranes were equally aged. Both the process of extracting wax and the long-term storage of skins will result in excessive loss of any solvent they may contain. The waxes used in 1945 may well have been changed by losing their solvents so as to have only the properties of the outer layer, and a repetition of these experiments in the light of present findings may show that they confirm our ideas.

THE TRANSITION PHENOMENON

It is tempting merely to point to the theory of mono-molecular organization and disorientation which explains events in the cockroach grease, and to suggest that similar processes occur in hard waxes. There are, however, very important differences between hard waxes and cockroach grease. Because of the mobility conferred by the solvent, cockroach grease can reform waterproof monolayers after temporary exposure to heat; it can spread over water, wet surfaces, and, we presume, over the entire surface of the insect. Hard waxes, so far as we know, can only spread at temperatures a few degrees below their melting-points (Beament, 1945); their properties are irreversibly changed by exposure to higher temperatures, and indeed we do not know whether in fact they do form a continuous layer over the surface of the animal. We can argue that unless the whole surface of the animal were covered with the hard wax it could not be as waterproof as our measurements indicate. Since our figures include spiracular loss, the cuticles of animals described in this paper are actually more impermeable than our figures would suggest.

But there is a more telling argument against the discontinuous distribution of these hard waxes over the cuticle surface; and it leads to an important conclusion about the transition of hard waxes. Suppose that most of the water transpired through these cuticles passed through numerous small unwaxed areas, large capillaries, etc. By the well-known pinhole diffusion theory of Brown & Escombe (1900) the contribution of small perforations to water loss will be out of all proportion to their summed area. When the waterproofing material is taken past its melting-point these areas must become covered by flow of the molten wax; indeed the reflexion electron micrographs of Holdgate & Seal (1956) actually show that the bloom of the *Tenebrio* pupa exposed to 80° C. does melt and flow into a layer over the surface. Yet the effect of exposure to temperatures above the transition point and above the melting-point is always to *increase* the permeability of the wax at *all* temperatures, as well as to remove the transition discontinuity. So we cannot associate transition with the microscopic redistribution of wax through melting; transition must surely be a molecular phenomenon.

We must next consider the shape of the permeability/temperature curves. Spiracular losses are included but it can be argued that they cannot affect the shape of the curves; losses from the equivalent of a free water surface must give a curve parallel to the abscissa when plotted on the axes used in these figures (see Beament, 1958*a*, p. 496); and we cannot expect to distinguish between losses from a waxed tracheal surface and from the exposed waxed cuticle itself. Over the limited range of temperature where experiments were possible (limited, to curtail evaporation of solvents) cockroach grease has a small and constant temperature coefficient of permeability, both below and above transition. In the natural state, hard wax also exhibits a constant temperature coefficient; once it has been permanently changed by heating, the temperature coefficient apparently obeys complex exponential laws, as is the case for most substances. Heat-treated wax has no very remarkable property, then, but the naturally occurring layers have, and the degree and nature of the difference between the two underlines the peculiar state in which the natural waxes occur.

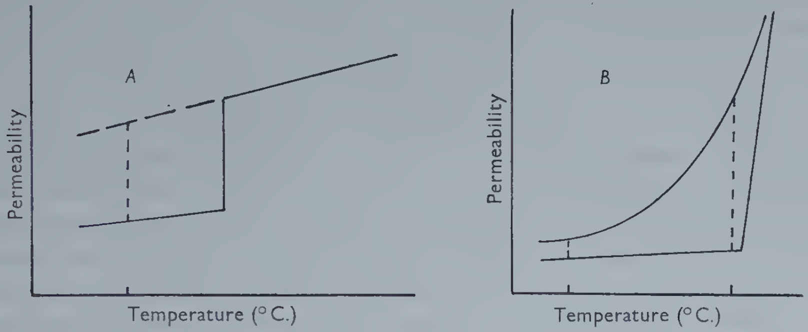


Fig. 16. Diagram showing (*A*) the form of the permeability/temperature curve for *Periplaneta* (Beament, 1958*a*). The permeability of the grease at the indicated temperature can be compared with the probable permeability of that grease when modified above transition, by projection of the upper curve. *B*, Typical permeability/temperature curves for insects with hard waxes; the ratio of the permeabilities of waxes in the natural state and after exposure to high temperatures depends on the temperature at which comparison is made. For further explanation, see text.

The peculiarity of cockroach grease below its transition temperature is the existence of the organized monolayer. By projecting (Fig. 16*A*) the part of the curve which represents completely disorganized grease down to temperatures where that same grease would develop the monolayer, it is possible to compare the properties of the grease in either state *at the same temperature* and thus to deduce the absolute permeabilities of the grease in monolayer and in disorientated states respectively. In characteristic curves for these hard waxes (Fig. 16*B*) the actual permeabilities of the natural state and the heat-treated (heat-stable) state can be compared. The magnitude of the change in permeability depends on the temperature at which comparisons are made; at temperatures just below the transition point the difference in permeability between the two forms of wax is so great that we can hardly attribute it merely to a change in a monolayer of a thick wax—it must surely be a change throughout the thickness of the layer. Some support for this argument can be obtained by calculating the absolute permeabilities of cuticles having hard waxes, and comparing the figures with that for the cockroach. In doing this we must bear in mind: (1) that the thickness of the wax is known only roughly for some of the insects—and here we shall assume throughout that it is 0·25 μ (Beament, 1945); (2) that the values we calculate will be high because they are based on figures which include spiracular loss; and (3) that the surface areas of the animals have been determined only very approximately, in most cases using formulae (Wigglesworth, 1945) which relate area to weight. With all these approximations and inaccuracies detailed comparison of the permeability of different species is not profitable; comparison of figures all obtained for one species is of course altogether more satisfactory.

The absolute permeability (given in ml. $\times 10^{-8}$ water vapour at N.T.P. per second referred to 1 cm.^{2} of material of thickness 1 ml. subjected to a pressure difference of 1 ml. of mercury) of disorganized cockroach grease at 25° C. is approximately 2·0. The waterproof monolayer of the cockroach has an order of permeability of 0·4 absolute unit, and the whole cuticle at this temperature, 1·65 units. Corresponding figures at 25° C. for the very waterproof insects in the present study are: *Tenebrio* mature pupae, 0·15; *Pieris* mature pupae, 0·5; *Rhodnius* nymphs, 0·9. These figures invite comparison with the value for the monolayer of cockroach grease, and so support our contention that molecular organization causing a very waterproof state extends through the wax layer beyond the confines of a single monolayer. After these three insects have been heated through only the lower transition point the permeabilities (at 25° C.) are respectively 0·75, 0·9 and 1·5 absolute units. The order of these figures is more in keeping with the permeability of the whole cockroach system—a monolayer and much disorganized wax; according to our present ideas they are the permeabilities of cuticles in which the natural state of the primary layer has been destroyed but which still have a thin natural layer on the cement. After heating through both transition points the absolute permeabilities at 25° C. rise respectively to 1·3, 1·9 and 2·4, suggesting that now all the wax is disorganized. If we next consider newly moulted pupae, *Tenebrio* has an absolute permeability of 0·4 and *Pieris* of 0·7; both figures suggest that the primary wax alone is much more extensively organized than the grease of the cockroach.

On the other hand, less waterproof arthropods, such as *Ixodes* and the larvae of *Calliphora* and *Pieris*, give values of absolute permeability between 3 and 6, and the permanent increase measured at 25° C. after heat-treatment is comparatively small. We can suggest that they resemble the cockroach more closely in the degree to which their waterproofing layers are naturally organized, but so many factors affect our calculations that we must await the outcome of much further research before this picture is complete.

There remains the inevitable question of the biological importance of a double waterproofing system. The outer layer over the cement is remarkably delicate (to handling and to adsorption by dust) and we have an anomaly so far unexplained. On being handled or dusted, animals having cement may show a considerable increase in their actual water loss but no reduction in the sharpness of the lower transition point (Figs. 14 and 15). The increased water loss is much greater than ought to be expected merely as a result of removing an outer monolayer but leaving a primary wax layer intact. It is interesting here to refer to the explanation which has been offered for the asymmetric permeability of insect cuticle (Beament, 1954).* When water was placed on the outside of isolated cuticle and dry air on the inside, high rates of water transport were recorded; this was explained by the great attraction for water of the hydrophilic part of the cuticle when it was dry; this attraction set up a large gradient across the wax. When the outer wax of a wax-cement-wax sandwich is removed (as by dust or abrasion) the cement is then excessively dried by the air, and greatly increased rates of flow are observed; there is a close parallel in behaviour between the cuticle model and the wax-cement system. Of course, the experimental findings in the present paper are far more firmly based than is this entertaining theory, and other explanations may be forthcoming; but, on the evidence of experiments alone, there is no doubt that the insect which protects its primary wax with a cement does achieve far greater impermeability if it has further wax above the cement.

CONCLUSION

It would appear that some degree of special molecular organization, and some degree of consequent abnormal impermeability, exists in the waterproofing waxes of a large number of terrestrial arthropods. Hardly anything is known of the physical chemistry of waxes composed of mixed molecular species, and we have little evidence on which to base speculation on the kind of molecular organization which may occur. The waterproofing system of the cockroach is unusual because its grease is mobile; the evidence that it has a monolayer of special properties in that grease is strong, but it is not necessarily true that the wax of an extremely waterproof insect such as the pupa of *Tenebrio* is naturally arranged in a monolayer or in a series of monolayers. On the other hand, the phenomena which occur at particular temperatures are so sharply defined that the wax must be behaving as a unit despite its heterogeneous composition; this once more points to the very special nature of the waterproofing wax. All our evidence, too, is compatible with the presence of solvents or volatile materials of lower molecular weight when these harder waxes are first secreted. The existence of such solvents makes it more easy to understand the migration of wax through the cement and the formation of special molecular arrangements in the wax; and this may have implications on the nature of the transport processes at an earlier stage still—when the waxes are metabolized and secreted.

It is to be doubted if techniques which measure permeability to water can alone take our knowledge of this waterproofing system very much further. It is more than ever necessary now to bring to the study of special organization in these waxes techniques divorced from permeability measurement.

SUMMARY

1. The relationship between the temperature of the cuticle and its permeability to water has been determined for a number of different terrestrial arthropods.

---

* In that paper the second fraction of the equation on p. 109 has been printed inverted.

2. In most of these animals the waterproofing wax loses its great impermeability to water very abruptly at a particular transition temperature; that temperature varies appreciably with the individual, but appears to be characteristic of any one species at a particular age within a particular instar. No change in permeability with temperature was found in *Tipula* sp. taken from soil, in which the cuticle was greatly damaged; above 48° C. the permeability of the cuticle of *Schistocerca* nymphs changes rapidly with increasing temperature, but this change is not abrupt.

3. In the permeability/temperature curves for larvae of *Pieris*, *Tenebrio*, *Calliphora* and *Nematus*, pupae of *Calliphora* and adults of *Schistocerca*, *Ixodes* and *Ornithodorus* only one transition discontinuity is found. In all these animals the wax maintains almost constant permeability up to the point of transition, above which permeability increases very rapidly with temperature.

4. Immediately after moulting, *Rhodnius* nymphs and pupae of *Tenebrio* and *Pieris* also reveal only one transition point; this occurs at a comparatively low temperature. But when cement is secreted over the primary wax layer, a second and higher transition point is found which is independent of the first point. The lower transition temperature seems constant in position during the life of an instar, but the upper point occurs at progressively higher temperatures as the instar ages. The evidence suggests that these animals have two special waterproof layers of wax, the one with high transition temperature occurring on the outer surface of the cement. This outer layer could arise by the migration of wax from the primary layer (with lower transition temperature) through the cement to the outer surface, there to lose volatile components and waterproof the surface.

5. In every animal investigated a permanent increase in permeability is produced by heating a wax layer above its transition point; after such treatment no transition point can be found.

6. From calculations of the absolute permeability of the various wax layers, and from the behaviour of the wax, it is suggested that all epicuticular waxes are laid down with some special molecular arrangement and that in very waterproof insects this may extend throughout the thickness of the wax layers; in less waterproof insects only a portion of the wax layer (as in the cockroach) may be specially organized.

7. Very slight mechanical damage promotes increased transpiration through the cuticle. Stationary adsorptive dusts applied directly to a primary wax layer do not affect permeability; applied to wax above the cement, they promote rates of evaporation in excess of the value exhibited by the same cuticle having a primary wax layer alone. This phenomenon is discussed in relation to the distribution of water within the transpiring cuticle.

Once again one acknowledges the considerable debt to Drs R. H. J. Brown and K. E. Machin for their advice and help on technical and physical problems. Drs A. D. Lees, A. Milne and T. Weis-Fogh and Messrs H. C. Bennet-Clark and B. O. Gardener have kindly supplied insect material; I am grateful to the Magnesium Elektron Co. and the Distillers Company for gifts of metal and plastic and for

technical information. Finally, I must thank Prof. V. B. Wigglesworth and Dr A. D. Lees for their continuing interest and encouragement.

APPENDIX

A MODIFIED APPARATUS FOR MEASURING EVAPORATION FROM INSECTS

The apparatus used in the present paper is a modified form of that previously described (Beament, 1958*a*) and the essential points in modification can be seen from Fig. 17. The balance beam (*b*) is a complex lattice comprising two girders of Elektron metal separated by plastic struts. It is suspended (*s*) asymmetrically so as to detect more sensitively the weight changes occasioned by the evaporation of water from the specimen (*i*). A compensation mechanism has been introduced to counter most of the effect of temperature change on such an asymmetric system. The insect, with its skin in contact with one thermocouple (t_1), is supported on a fine nylon net across one end of the lattice; weight changes are measured by adjusting with a micrometer movement (*m*) the distribution of a gold chain (*g*) which is looped between the opposite end of the lattice and the spindle piston of the micrometer. Air temperature is measured and controlled via two thermistors (t_2,t_3) by the method of Beament & Machin (1958, 1959), but adapted to produce much greater heat dissipation in both the controlled (h_1) and the background (h_2) heaters. It was found necessary to enclose both heaters inside the fan housing and to place the fan (*f*) *under* the heaters; the direction of air circulation is thus reversed as compared with the apparatus previously described. The thickness of the plastic box for the balance has been increased to $\frac{1}{4}$ in. for better insulation, and massive brass bars have been added to prevent distortion at high temperatures. The box measures 3 in. × 3 in. × 12 in. and is sealed with a neoprene gasket. Air or nitrogen (see p. 401) was obtained dry and compressed in cylinders and could be introduced via a tap in the base of the box. Further information can be obtained from photographs in the paper by Beament (1958*b*).

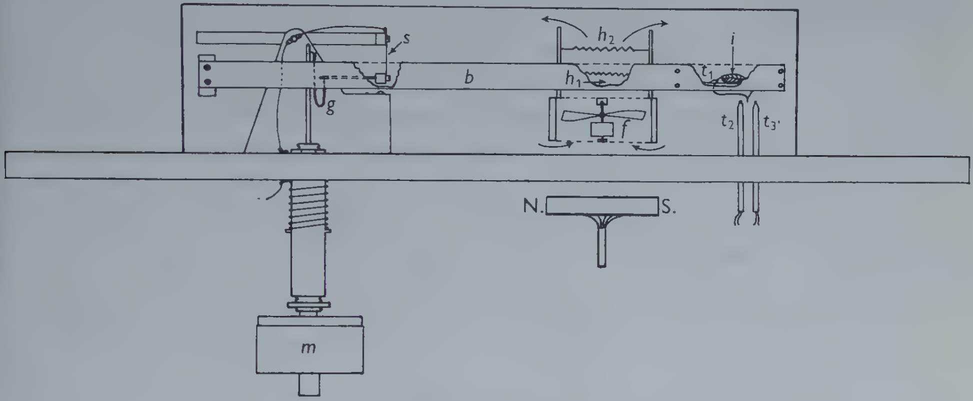


Fig. 17. Schematic drawing of balance used in the investigation. For explanation, see text.

SPECIFICATION OF EQUIPMENT

Maximum weight of specimen: 3 g. Accuracy of weight change: ±0·01 mg. Temperature measurement and control: ±0·04° C. over range, ambient −40° C.; ±0·08° C. over range 40–80° C. Water content of air: 5 p.p.m. Temperature coefficient of balance: a change equivalent to an apparent alteration at the specimen of 2 mg. was produced by raising the temperature from 20 to 80° C. This was linearly distributed over the range and all readings have been appropriately corrected; the change is independent of load, primarily because riders and specimen are on the same side of the fulcrum. No drift could be detected under conditions of constant temperature, so that measurements of evaporation by weight change are absolute readings.

REFERENCES

Beament, J. W. L. (1945). The cuticular lipoids of insects. *J. Exp. Biol.* **21**, 115.

Beament, J. W. L. (1946). The waterproofing process in eggs of *Rhodnius prolixus* Stahl. *Proc. Roy. Soc.* B, **133**, 407.

Beament, J. W. L. (1948). The role of wax layers in the waterproofing of insect cuticle and egg-shell. *Disc. Faraday Soc.* **3**, 177.

Beament, J. W. L. (1951). The structure and formation of the egg of the fruit-tree red spider mite, *Metatetranychus ulmi*. *Ann. Appl. Biol.* **38**, 1.

Beament, J. W. L. (1954). Water transport in insects. *Symp. Soc. Exp. Biol.* **8**, 94.

Beament, J. W. L. (1955). Wax secretion in the cockroach. *J. Exp. Biol.* **32**, 514.

Beament, J. W. L. (1956). Physico-chemical and physiological problems associated with the mechanism of toxicity of insecticides. *2nd Int. Conf. Plant. Protection.*

Beament, J. W. L. (1958*a*). The effect of temperature on the waterproofing mechanism of an insect. *J. Exp. Biol.* **35**, 494.

Beament, J. W. L. (1958*b*). Why an insect is so waterproof. *New Scientist*, **4**, 847.

Beament, J. W. L. & Machin, K. E. (1958). British Prov. Pat. Spec. No. 5184/58.

Beament, J. W. L. & Machin, K. E. (1959). A new method for the regulation of air temperatures. *J. Sci. Inst.*

Brown, H. T. & Escombe, F. (1900). Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. *Phil. Trans.* B, **193**, 223.

Edney, E. B. (1957). The survival of animals in hot deserts. *Inaugural Lecture, Univ. Coll. Rhodesia & Nyasaland.* Oxford University Press.

Holdgate, M. W. & Seal, M. (1956). The epicuticular wax layers of the pupa of *Tenebrio molitor* L. *J. Exp. Biol.* **33**, 82.

Kramer, S. & Wigglesworth, V. B. (1950). The outer layers of the cuticle in the cockroach, *Periplaneta americana*, and the function of the oenocytes. *Quart. J. Micr. Sci.* **89**, 291.

Lees, A. D. (1947). Transpiration and the structure of the epicuticle in ticks. *J. Exp. Biol.* **23**, 291.

Malek, S. R. A. (1958). The appearance and histological structure of the cuticle of the desert locust, *Schistocerca gregaria* (Forskål). *Proc. Roy. Soc.* B, **149**, 557.

Way, M. J. (1950). The structure and development of the larval cuticle of *Diataraxia oleracea* (Lep.). *Quart. J. Micr. Sci.* **91**, 145.

Wigglesworth, V. B. (1945). Transpiration through the epicuticle of insects. *J. Exp. Biol.* **21**, 97.

Wigglesworth, V. B. (1947). The epicuticle of an insect, *Rhodnius prolixus* (Hemiptera). *Proc. Roy. Soc.* B, **134**, 163.

Wigglesworth, V. B. (1948). The structure and deposition of the cuticle in the adult mealworm *Tenebrio molitor* (Coleoptera). *Quart. J. Micr. Sci.* **89**, 197.

Wigglesworth, V. B. (1958). Abrasion of the insect cuticle by aqueous suspensions of small particles. *Nature, Lond.*, **181**, 779.

Wolfe, L. S. (1954). The deposition of the third instar larval cuticle of *Calliphora erythrocephala*. *Quart. J. Micr. Sci.* **95**, 49.

THE HYDRAULIC MECHANISM OF THE SPIDER LEG

By D. A. PARRY and R. H. J. BROWN
Department of Zoology, University of Cambridge

(*Received* 12 *February* 1959)

INTRODUCTION

Hinge joints occur in the legs of various arthropods, notably *Limulus* (Snodgrass, 1952); arachnids; diplopods, chilopods and pauropods (Manton, 1958*b*). At a hinge joint flexion is muscular, but the anatomical form of the joint rarely permits the presence of antagonistic extensors (see Manton, 1958*a*, fig. 151 for an exception). In the recent account of the leg muscles of the house spider *Tegenaria* sp., Parry (1957) suggested that extension might be due to hydraulic forces in the leg. Manton (1958*b*) attributed to the same cause the full extension of the arachnid leg when the limb tip is off the ground but considered that extension during a propulsive stroke was passive and due to the ground reaction on the limb and so, indirectly, to depressor muscles.

In the present paper support is given to the idea that active extension occurs at the hinge joints of the *Tegenaria* leg and that the mechanism is a hydraulic one. First, we measure the internal pressure in the leg of intact spiders and find it to be surprisingly high. Secondly, we establish an empirical relation between the internal pressure of the leg and the torque at the hinge joints. Thirdly, we measure the torques actually developed at the hinge joints when a spider accelerates a mass attached to the leg, and use the relation between torque and pressure to show that the measured torques can be accounted for by the pressures which occur in the leg.

MEASUREMENT OF INTERNAL PRESSURE

Methods

The pressure inside the leg of an intact living spider can be measured by taking advantage of the thin flexible articular membranes at the two hinge joints. This principle is employed in two ways:

(*a*) *Direct observation of the membrane* (Fig. 1). A sleeve is sealed over the leg and the membrane observed through a binocular microscope. To measure the *maintained resting pressure*, the pressure in the sleeve is slowly raised until the membrane just collapses. To measure *transient pressures* developed when the spider is struggling, the sleeve pressure is raised to a predetermined value above the resting pressure so that the membrane collapses, and the spider is then stimulated. The reinflation of the membrane indicates that the internal pressure equals or exceeds the sleeve pressure.

(*b*) *Use of a high impedance transducer.* The Cambridge Instrument Company very kindly lent us one of their photo-electric transducers and associated equipment. The principle of this instrument is illustrated in a purely formal way in Fig. 2. The stiffness of the diaphragm is such that the instrument is almost isometric, a pressure change of 1 cm. Hg producing a volume change of 5×10^{-3} mm.^{3} (cf. the joint volume of the spider which is about 0·5 mm.^{3}). One leg of a living spider is sealed into the cavity as shown, the stop-cock, *S*, is opened and a zero recorded. The pressure is then raised to a pressure *P*, above the internal resting pressure of the spider, so that the articular membrane collapses. The stop-cock *S* is then closed and the recorder started. The spider is stimulated with a paint brush or a puff of air and the excess of internal pressure above *P* is thus recorded against time.

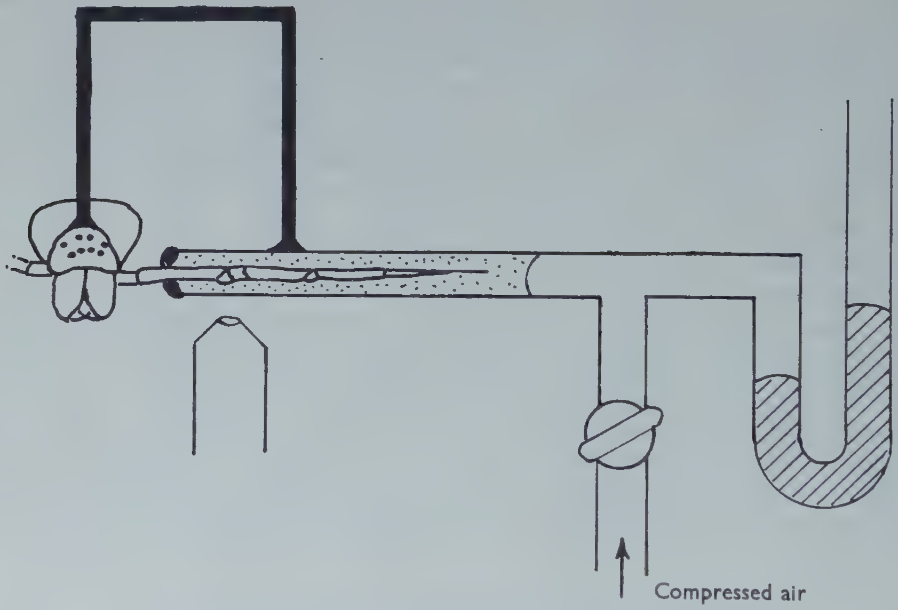


Fig. 1. Method of mounting a spider for observation of the articular membrane during the application of external pressure.

RESULTS

Resting pressures. When a spider is immobilized with one leg in a sleeve, it maintains a positive pressure of approximately 5 cm. Hg which we call the resting pressure. The following figures (each a mean of four successive determinations) indicate the sort of variation encountered in different spiders: 4, 5, 5, 8, 9, 11, 11 cm. Hg. In the absence of other evidence we presume that these pressures are typical of the resting spider when supporting its own weight in normal life.

Transient pressures. When a spider, mounted as indicated in Fig. 2, is stimulated by a puff of air or by a paint brush the internal pressure rises to several times the resting value and then falls again in a time which rarely exceeds 1 sec. and is usually about $\frac{1}{5}$ sec. A transducer record showing an unusually high transient pressure is shown in Fig. 3. The peak pressures which have been recorded in this way are as follows:

Spider *A*	11, 15, 17, 17, 19, 20, 22, 23, 24 cm. Hg
Spider *B*	16, 24, 27, 39, 39+ cm. Hg
Spider *B* (next day)	16, 20, 23, 24, 25, 25, 45 cm. Hg
Spider *C*	25, 25, 30, 30, 40 cm. Hg

It will be seen that pressures of up to 45 cm. Hg have been recorded.

Comparable results have been obtained by the simpler method (*a*) described above. The maximum sleeve pressures against which a spider was observed to re-inflate its articular membrane was 40 cm. Hg. This was repeated on the same spider on two successive days.

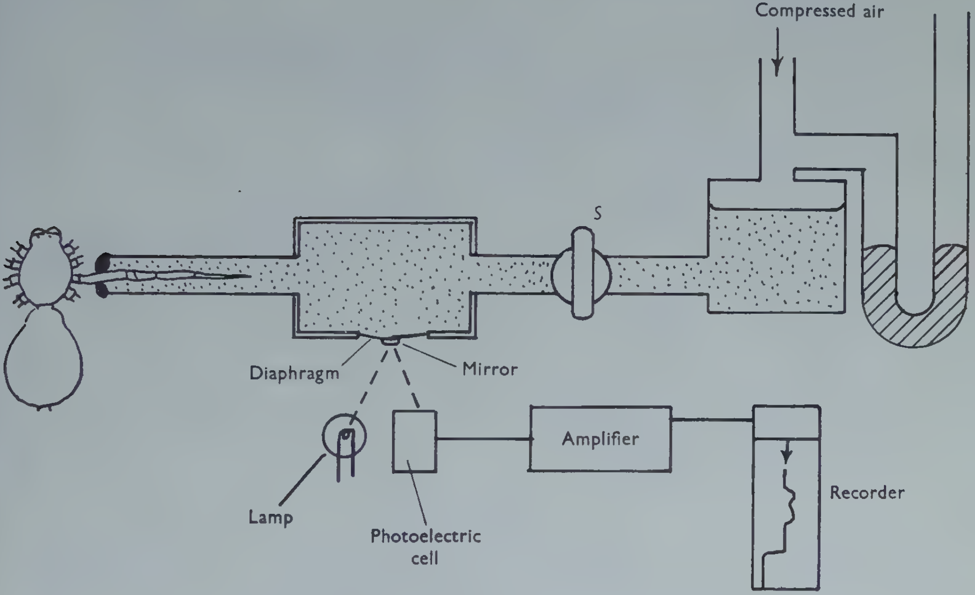


Fig. 2. Measurement of transient pressures in the leg of a spider using a high impedance photo-electric transducer.

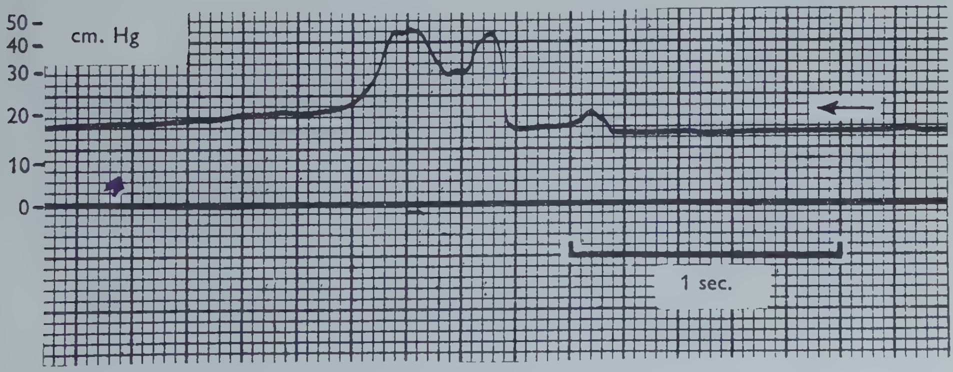


Fig. 3

RELATION BETWEEN PRESSURE AND TORQUE AT A JOINT

Direct determination

A leg is removed at the coxa-trochanter joint where normal autotomy occurs (Parry, 1957) and waxed into the end of a Perspex tube with a 1:1 beeswax-resin mixture Krogh & Weis-Foch, 1951). The tip is cut off and the leg perfused with 1·3% magnesium chloride to relax the muscles. The tip is then sealed with wax and the Perspex tube mounted in the way shown in Fig. 4, thus enabling the relationship between internal pressure and torque at the joint to be determined under static conditions for different joint angles.

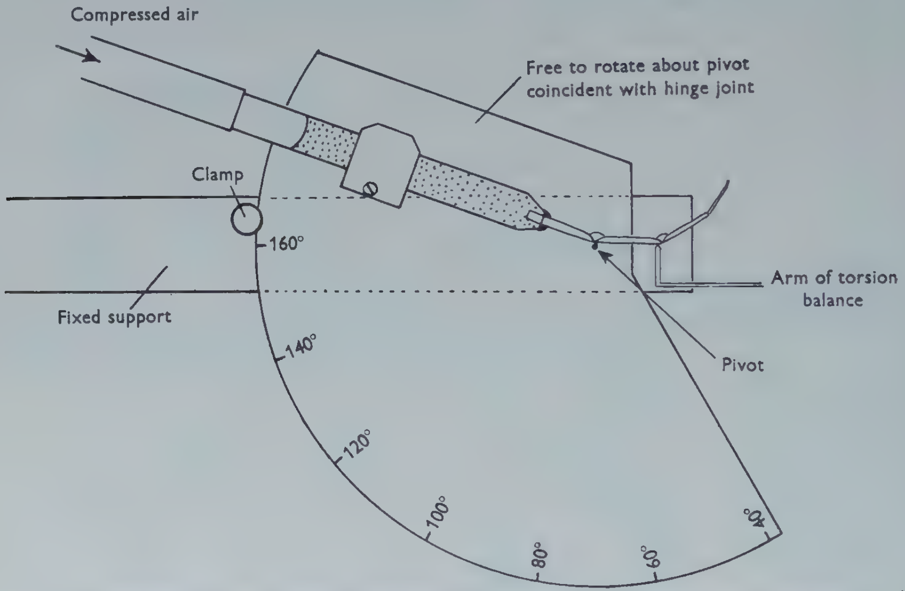


Fig. 4. Measurement of the relation between the internal pressure of the leg, the joint angle and the torque developed at the hinge joint.

Fig. 5 shows the relationship between pressure and torsion balance reading for the femur-patella joint ('upper joint') and the tibia-basitarsus joint ('lower joint') of a particular leg whose size is indicated by the fact that the distance between the two joints was 11 mm. The distance between the upper joint and the torsion balance pivot was 10 mm.; that between the lower joint and the pivot was 8 mm. For a given joint angle (θ) the relation between pressure (P) and torque (C) is linear, that is $C = k_\theta P$, where k_θ decreases as θ increases. If C is expressed in dyne-cm. and P in dynes/cm.^{2}, k_θ has the dimension cm.^{3} and its value for the selected values of θ is shown in the figure.

Indirect determination

It can be shown that if the torque C is due to the internal fluid pressure, k_θ is numerically equal to $\Delta V/\Delta\theta$, where ΔV is the small change in the volume of the joint due to a small change of angle $\Delta\theta$. The relation between ΔV and $\Delta\theta$ is measured,

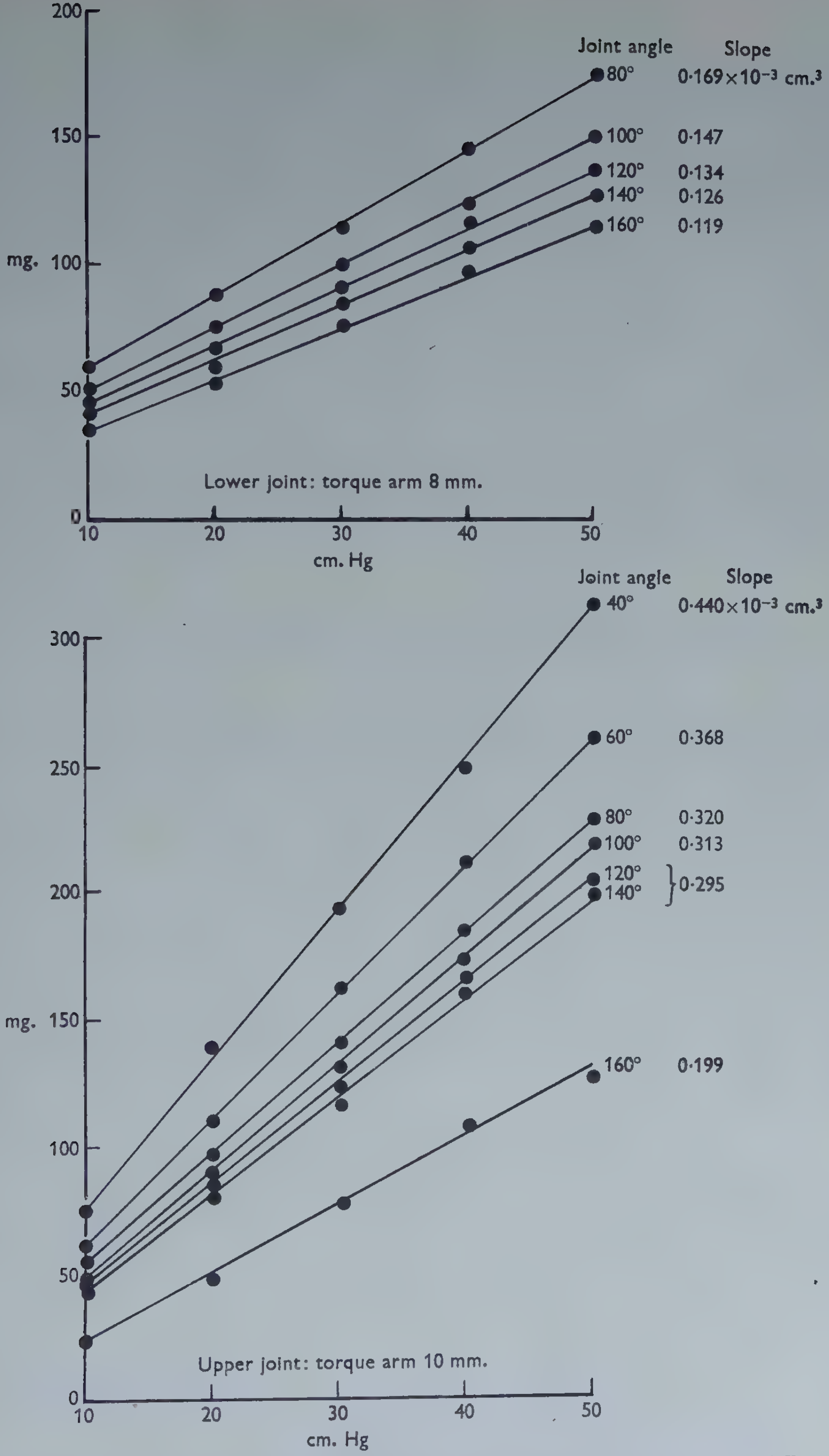


Fig. 5. Relation between the internal pressure and the torsion balance reading for different joint angles. The 'torque arm' is the distance between the joint and the arm of the balance (see Fig. 4).

using the apparatus shown in Fig. 6. At a given pressure the joint angle is increased in 20° stages from 40° to 160° and the movement of the bubble is measured with a travelling micrometer. A second series of readings is taken as the angle is decreased, and a mean for each angle is determined. In the event, the difference between the ascending and descending readings was negligible.

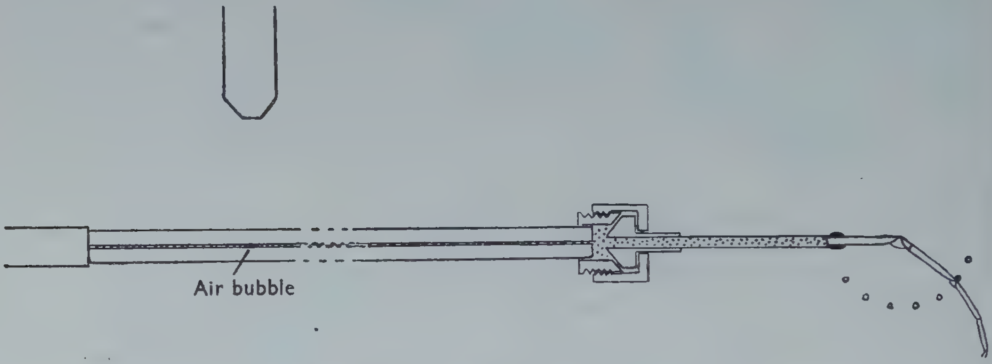


Fig. 6. Measurement of the relation between a change of joint angle and the consequent change in joint volume.

The results shown in Fig. 7 were obtained from the same leg as those of Fig. 5 and so k_θ ($= C/P$) can be directly compared with $\Delta V/\Delta\theta$:

Joint angle		40°	60°	80°	100°	120°	140°	160°
Upper joint	C/P	0·44	0·37	0·32	0·31	0·30	0·29	0·20 mm.^{3}
	$\Delta V/\Delta\theta$	—	0·31	0·29	0·27	0·26	—	— mm.^{3}/radian
Lower joint	C/P	—	—	0·17	0·15	0·13	0·13	0·12 mm.^{3}
	$\Delta V/\Delta\theta$	—	0·20	0·18	0·15	0·14	0·13	— mm.^{3}/radian

The agreement between the direct and indirect determinations of k_θ is good, although there is a small consistent discrepancy in the measurements of the upper joint. In the next section the values for k_θ obtained by direct measurement are used.

FORCES DEVELOPED DURING EXTENSION

The extension torques developed at the hinge joints in normal walking are those producing a force at the foot (likely to be greatest in the fourth pair of legs which push forwards) together with those associated with accelerating and raising the distributed mass of the leg (likely to be greatest in the first pair of legs which straighten forwards). No attempt has been made to measure these torques. Instead, the leg of an intact living spider was loaded with additional mass in the form of a brass ring of 45 mg. and photographed by high-speed cinematography (Brown & Popple, 1955) while it was extending in a horizontal plane about the upper hinge joint. Analysis of the film provides the angular acceleration of the leg, from which the torque developed at the hinge joint can be determined. The pressure needed to produce this torque can be calculated from the data given in the last section and can in turn be compared with the pressures which have actually been measured in living spiders.

This method of measuring the torque is only a rough one: no account can be taken of internal friction (including tension in the flexor muscles); and a frame interval of 6 msec. is not short enough to give an accurate measure of acceleration.

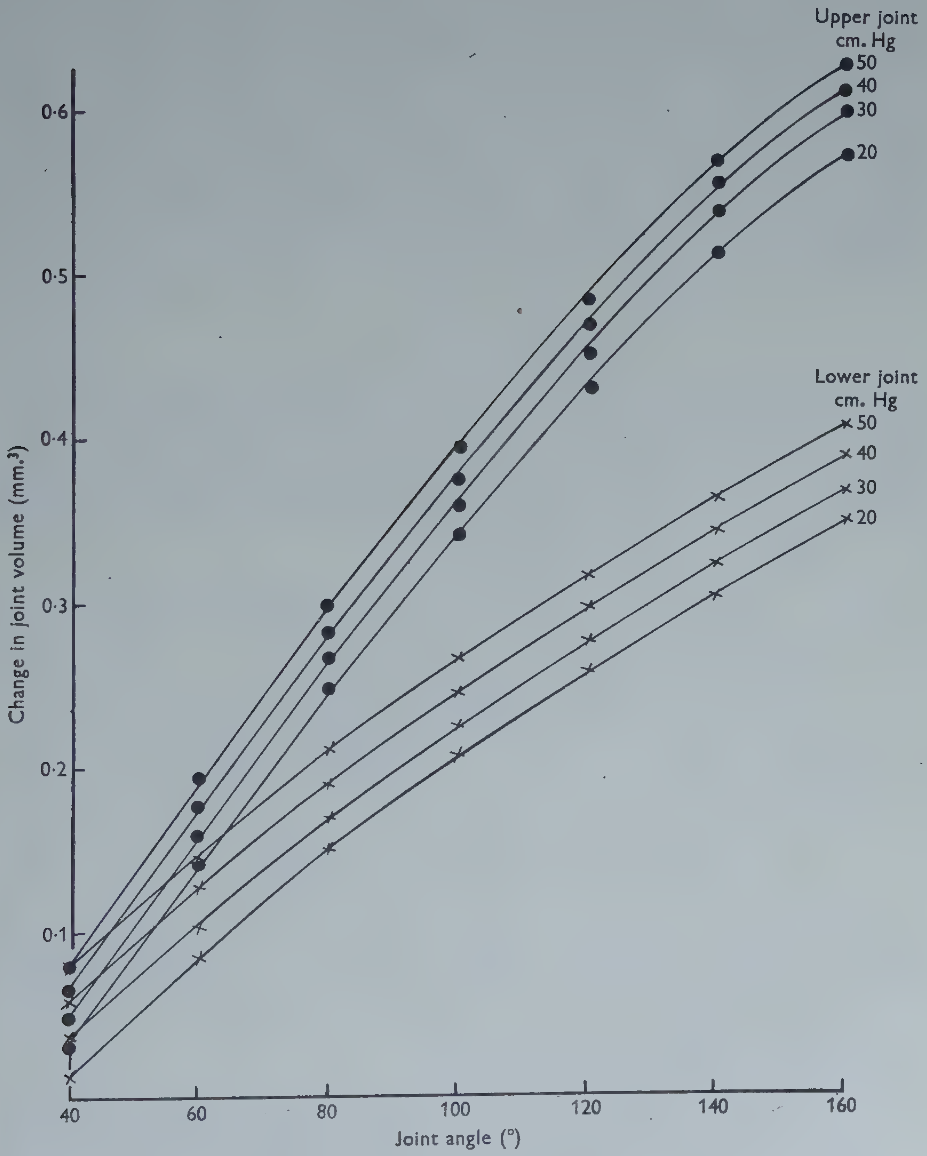


Fig. 7. Relation between changes in joint angle and changes in joint volume at different internal pressures.

From four sequences the following example is selected as involving the greatest acceleration which has been encountered:

t	0		6		12		18	msec.
Angular velocity (ω)	0		20·0		47·5		50·4	radian/sec.
Angular acceleration ($\dot{\omega}$)		3·3		4·6		0·47		$\times 10^3$ radian/sec.^{2}
Torque ($I\dot{\omega}$)		125		174		17·8		dyne-cm.
Pressure (C/k_θ)		31		45		5		cm. Hg

In the other three sequences obtained (two different spiders) the maximum torques developed during a 6-msec. period, and the corresponding pressures, were: 130 dyne-cm. (35 cm. Hg); 77 dyne-cm. (18 cm. Hg); and 54 dyne-cm. (13 cm. Hg). It will be seen that the pressures required to produce these torques all lie within the range of pressures which the spiders have been shown to develop. The figures furthermore establish directly that the torques developed at the hinge joints can be considerably greater than are required merely to extend the leg when it is off the ground (cf. Manton, 1958*b*).

DISCUSSION

The hydraulic mechanism

Anatomy. In the spider, prosoma and abdomen are separated by a narrow pedicel (Fig. 8). The prosomal tergum and sternum are relatively rigid plates—the one convex, the other flatter—connected together by an unsclerotized lateral region. The legs are inserted into this lateral region and are without articulations with either tergum or sternum, which are therefore free to move apart or come together (see Fig. 8). According to Brown (1939) there are no tergo-sternal muscles, but dorso-ventral muscles run from the tergum to the endo-skeleton, the stomach and the proximal ends of the coxae of the legs.

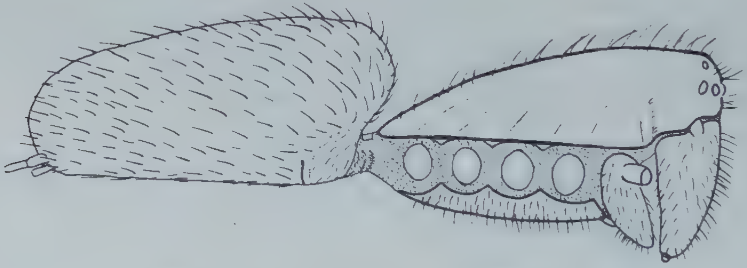


Fig. 8. *Tegenaria atrica* showing the flexible lateral region which intervenes between prosomal tergum and sternum.

The abdomen is relatively flexible and becomes wrinkled and apparently shrunken when an animal is short of food or water. Millot (1949) describes a thin subcuticular muscle layer; otherwise the abdominal musculature is scanty (Brown, 1939). The heart lies in the abdomen and a single large aorta runs through the pedicel into the prosoma where arteries distribute the blood peripherally.

Resting pressure. Under the conditions of our experiments the haemocoelic pressure in the legs of unstimulated spiders is about 5 cm. Hg and we suggest that the normal resting pressure is of approximately this value. Regarding the production and distribution of this pressure there are three possibilities:

(1) The pressure might be uniformly distributed throughout the prosoma and abdomen and due to muscle tension. This seems unlikely because the isolated abdomen is found to become grossly distended at such a pressure and yet its musculature appears inadequate to maintain the characteristic shape in life. It should be noted that if the prosoma and abdomen were at the same pressure no load would be imposed on the heart, whose systolic pressure need only be sufficient to overcome the peripheral resistance.

(2) The pressure might be limited to the prosoma, the abdomen being at atmospheric pressure. This accords with the observed lack of turgidity in the abdomen but demands that the systolic pressure of the heart should just exceed the prosomal pressure, thus developing considerably more pressure than is usual among arthropods (compare data given in Prosser (1950), Table 67; but note also Harris & Crofton (1957) on the nematode *Ascaris* in which the mean internal pressure is 7 cm. Hg).

There is a little evidence that the heart may in fact produce the required high pressure. The blood flow in a spider's leg can be seen by transmitted light if the leg is sealed into a flat-sided cell filled with a physiological solution (1 l. of spider Ringer is made up with: 144 c.c. M/15 KH_2PO_4; 144 c.c. M/15 Na_2HPO_4; 347 c.c. 0·54M NaCl; 5·56 c.c. 0·36M $CaCl_2.6H_2O$; 5·56 c.c. 0·36M $MgCl_2.6H_2O$).

If the prosoma is now compressed dorso-ventrally the blood flow in the leg eventually stops. The haemocoelic pressure at which this occurs can be measured in the usual way by determining the external pressure which has to be applied to the leg in order just to collapse the articular membrane. The pressures thus measured will presumably represent the maximum which the heart can produce. Readings were as follows: 10·4, 10·6, 11·2 and 11·6 cm. Hg.

Clearly this needs further investigation, but so far as the available evidence goes, it suggests that the 'maintained pressure' of 5 cm. Hg occurs in the prosoma, but not the abdomen. This being the case it might be expected that some form of valve or sphincter would occur in the pedicel and would play an important part in regulating prosomal pressure.

(3) A third possibility should be mentioned: that the pressure is limited to the legs alone. This would still demand a high heart pressure, and would involve a sphincter at the base of the leg and suitable muscles in the leg, of which there are no signs.

Transient pressure. Pressures of up to 45 cm. Hg, maintained for a fraction of a second, have been measured on a stimulated spider. If two legs are separately sleeved and subjected to the same external pressure, the articular membranes are found to inflate and deflate simultaneously when the spider is stimulated. This suggests that the transient pressure arises in the prosoma rather than in the individual legs (where, as noted above, no muscles seem suitably disposed to produce it) and it may well be due to the sudden activation of the prosomal muscles and slight dorso-ventral compression. This sudden rise of pressure would, of course, temporarily stop the prosomal circulation, and in this connexion it is interesting to note the well developed aortic valve in the pedicel (see, for example, Millot, 1949, fig. 419, based on the beautiful work of Schneider, 1892).

Dynamic considerations. By adding mass to the leg of a living spider and finding the acceleration during an extensor movement we have measured the torques which can be developed at a hinge joint. We have then calculated the internal pressure needed to develop these torques, using values for k_θ empirically determined. However, k_θ was measured under static conditions and it remains to be seen whether it can be applied under dynamic ones. Two considerations arise:

(1) When a hinge joint is extending fluid must flow into the joint thus setting up a pressure gradient in the leg so that the pressure in the joint will not be the same as the pressure in the prosoma. A rough application of Poisseuille's equation shows that this gradient may be insignificant. The maximum observed rate of extension of the upper hinge joint was about 50 radian/sec. at a joint angle of about 90°. This is equivalent to a volume change of 15 mm.^{3}/sec. Taking the viscosity of the blood to be 0·05 dyne-sec./cm.^{2} (as in human blood—no direct measurements are available), and the length of the upper part of the leg to be 1 cm., we get:

$$r^4 \Delta p = 0{\cdot}002 \text{ dyne-cm.}^2,$$

where r = radius of duct and Δp = pressure difference between prosoma and upper hinge joint. Now as the channel is of indeterminate shape no precise value can be assigned to r; but it can be seen that Δp is negligible provided that the equivalent duct is not less than 0·02 cm. radius, or two-fifths of the actual radius of the femur, which is not unreasonable. However, the matter needs further investigation.

(2) If the prosoma were very indistensible then the withdrawal of a small quantity of fluid would produce a significant fall in pressure. But in the section on anatomy we have pointed out that the prosoma is built in such a way that changes in volume can readily be produced.

Bleeding. It might be thought that, owing to the high pressures occurring in the spider, bleeding would be a serious hazard, and it is the case that spiders readily die from this cause. Perhaps the most frequent form of damage is the loss of a leg and here a special protective mechanism has been evolved (Parry, 1957). But minor damage to the prosoma need not be fatal; the withdrawal of blood together with the relaxation of the prosomal muscles would produce an immediate fall in pressure and the wound might heal in a few days. During this time the spider is not immobilized, but we have no reason to think that ordinary walking necessitates the high pressures which we have measured in uninjured animals, especially if the hind legs (the only ones whose propulsive stroke is an extension) contribute less force than usual.

SUMMARY

1. The blood pressure inside the leg of the house spider *Tegenaria atrica* has been measured. Maintained pressures of about 5 cm. Hg and transient pressures of up to 40 cm. Hg have been found.

2. The relation between the blood pressure in the leg and the extension torque at the hinge joints has been established.

3. Considerable torques can be developed at the hinge joints during extension, for example, when accelerating a mass fixed to the leg. The transient pressures found to arise in the leg are adequate to account for these torques.

4. The hydraulic mechanism is discussed. The available evidence suggests that the pressure found in the legs occurs also in the prosoma but not in the abdomen, in which case the maintained pressure must be due to the heart. This, however, requires further investigation.

We are greatly indebted to the Cambridge Instrument Company for the loan of a photo-electric transducer; and also to many friends who have supplied us with spiders. The spider Ringer was based on determinations of freezing-point depression and sodium and potassium concentration of *Tegenaria* blood made for one of us some years ago by Dr P. C. Croghan and not hitherto published.

REFERENCES

Brown, R. B. (1939). The musculature of *Agelena naevia*. *J. Morph.* **64**, 115–66.

Brown, R. H. J. & Popple, J. A. (1955). Multiple flash photography. *Med. Biol. Illustr.* **5**, 23–8.

Harris, J. E. & Crofton, H. D. (1957). Structure and function in the Nematodes. *J. Exp. Biol.* **34**, 116–30.

Krogh, A. & Weis-Foch, T. (1951). Respiratory exchange of the desert locust. *J. Exp. Biol.* **28**, 344–57.

Manton, S. M. (1958*a*). Evolution of arthropodan locomotory mechanisms, Pt. 6. *J. Linn. Soc. (Zool.)*, **43**, 488–556.

Manton, S. M. (1958*b*). Hydrostatic pressure and leg extension in arthropods. *Ann. Mag. Nat. Hist.* Series 13, **1**, 161–82.

Millot, J. (1949). Ordre des Aranéides, in Grassé, P.-P. (directeur): *Traité de Zoologie*, **6**. Paris: Masson.

Parry, D. A. (1957). Spider leg muscles and the autotomy mechanism. *Quart. J. Micr. Sci.* **98**. 331–40.

Prosser, C. L. (ed.) (1950). *Comparative Animal Physiology*. Philadelphia: Saunders.

Schneider, A. (1892). Mélanges arachnologiques. *Tablettes Zoologiques*, **2**, 135–98.

Snodgrass, R. E. (1952). *A Textbook of Arthropod Anatomy*. New York: Comstock.

All communications should be addressed to the Editors,

The Journal of Experimental Biology, Zoological Laboratory,
Downing Street, Cambridge, England.

MANUSCRIPTS:

Authors are requested to present their work in as *concise* a form as possible. MSS. should be *typewritten* (double spacing) on one side only of the paper, and the pages numbered. *Tables* and *Legends* should be on separate sheets from the rest of the MS. and should be numbered. The place where they are to be inserted in the text should be indicated in the margin of the MS. *Bibliography* should be given under the heading of 'References' and in the following form: Surname of authors (in alphabetical order), initials, date of publication, title of paper, title of journal (abbreviated according to the *World List of Scientific Periodicals*), volume and pages of reference (first and last). In the text a reference should be quoted by the author's name and date (in brackets), and not by the numerical order of the paper in the list of references. Every paper must contain a summary of the chief results of the enquiry.

ILLUSTRATIONS:

(i) *Line blocks.* These should be used whenever possible. Illustrations should be pen drawings in Indian ink (*jet black and waterproof*) on smooth white Bristol board, heavy drawing paper or good quality tracing paper. Drawings must not be folded or creased in any way. *Lettering should be temporarily and lightly put in by the author in soft pencil.* Where considerable reduction is required it is essential that the drawing be of such a character as to bear such reduction. *All brush work, tinting or pencil shading is to be avoided.*

Charts and curves can often be drawn to best advantage on *graph paper ruled in pale blue.* The blue lines while ensuring accuracy, are easily eliminated by the printer, only the blackened lines that are desired remaining.

(ii) *Text half-tone blocks.* These are suitable for illustrations involving brushwork, or in which the depth of shading is an essential feature. They can be used for such things as oscillograph records and some photographs can be produced in this way, a good glossy bromide print being required. Illustrations should be gummed on *white* card, grouped and numbered as they are to appear in print. All lettering should be shown in position on a covering sheet of transparent paper.

(iii) *Plates.* Plates should be used only for illustrations, such as photomicrographs, in which the most accurate reproduction of fine detail is called for. Plates are expensive and the Editors may require an author to defray the cost of plates which in their opinion are not essential. The photographs making up the plate should be gummed on *white* card, grouped and numbered as they are to appear in print. Exclusive of margin the plate figures should not cover, when reduced, an area greater than 7½ in. in length × 5 in. in width when ready for reproduction as a single plate, or 7½ in. × 11½ in. in the case of double plates. All lettering should be shown in position on a covering sheet of transparent paper.

Authors are asked not to submit sheets of illustrations which are more than foolscap size; or, if this cannot be avoided, to include photographic reductions for the convenience of referees.

ABSTRACTS:

Authors should submit with their MSS. *four copies* (typewritten, double spacing) of an abstract suitable for biological abstracting journals. The abstract will not appear in the *Journal of Experimental Biology* but will be scrutinised by the Editors before being passed for publication. The summary of a paper may serve as an abstract provided that it conforms to the following requirements. The abstract should outline as briefly as possible the results and the definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words and the abstract should never exceed 3% of the original. An address (to which applications for offprints may be sent) should be added.

PROOFSHEETS AND OFFPRINTS:

Authors will recieve one set of slip proofs for correction and return to the Editors. A page proof will also be sent if the slip proof is marked by the author 'Revise'. An allowance of ten shillings per sheet of sixteen pages will be made for alteration apart from printer's errors. Authors may be charged for any excess over this average. Authors will receive 50 copies of their papers free; additional copies may be purchased and should be ordered when the proofs are returned to the Editors.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY

The Society holds Conferences four times a year at which the results of experimental investigations are presented and discussed. Enquiries about membership of the Society should be addressed to one of the Secretaries:

Dr J. D. CARTHY, Department of Zoology, Queen Mary College, London, E. 1
Dr G. E. FOGG, Botany Department, University College,
Gower Street, London, W.C. 1

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, CAMBRIDGE
(BROOKE CRUTCHLEY, UNIVERSITY PRINTER)